Genetic analysis of glial cells in Health and Disease using Drosophila melanogaster as a model

Mazaud, David

Awarding institution:
King's College London

Genetic analysis of glial cells in Health and Disease using *Drosophila melanogaster* as a model

David Mazaud

Thesis submitted for the degree of Doctor of Philosophy

March 2016

MRC Centre for Developmental Neurobiology
Maurice Wohl Clinical Neuroscience Institute, IOPPN
King's College London

Declaration of authorship

I, David Mazaud, hereby declare that:

This thesis and the work presented in it are my own and have been generated by myself apart from the below mentioned technical experiments:

- Catarina Pimentel (Post-doc) did the injection protocol of the Dextran dye and helped in the dissections and imaging of the fly brains and in the analysis of the data, presented in Figure 5.10, Chapter V.
- Adel Boudi (PhD student) did the retina embedding and sectioning for the tangential and horizontal sections, taken the images and helped in the analysis of the data, presented in Figure 5.11, Chapter V.
- Nadine Tüchler (Erasmus student under my direct supervision) provided a technical help in the isogenisation process for *mfas*, *caps* and *trn* RNAi lines and to do their corresponding lifespan.

Part of this work has been submitted for publication:

Trébuchet, G., Zsámboki, J., Mazaud, D., Lai, E., Fanto, M. and Giangrande, A. (2016). "Keeping the balance between glia and blood in the *Drosophila* embryo."

I also participated to the following work, submitted for publication:

Pardo, R.*, Mazaud, D.*, Baron, O., Boudi, A., Claverol, S., Gaultier, C., Oozeer, F., Bonneu, M., Fanto, M. (2016). "Hippo promotes Atg13 phosphorylation to regulate autophagy in neurons."

Abstract

The increasing understanding of neurodegenerative diseases at the cellular and molecular level has directed part of the research toward the investigation of the role of glial cells in the pathogenesis. Specific impairment of this long-time neglected cell type has revealed that it was much more than just glue and is now considered a major player in either triggering defects or in the progression of nervous system disorders. My thesis proposes to investigate the role of glial cells in disease condition but also in "healthy" brain in order to uncover new mechanisms important in the complex interactions between glia and neurons. Using glial expression of the polyglutamine Atrophin as a disease model, I first characterized the importance and the participation of each glial subtype in the reduction of the *Drosophila* lifespan. The use of different specific glial drivers to express the mutated protein showed that the astrocyte-like glia might be the most important cell type, but not the only one, involved in the decrease of the lifespan. Then, using an unbiased RNAi-based screening method, I modulated neuronal gene expression in a newly established *Drosophila* model with impaired glial functions via the expression of a newly engineered polyglutamine Atrophin. Lifespan was used as a read out for the impairment of the nervous system homeostasis, leading to the discovery of genes able to modulate the reduction of the lifespan due to glial toxicity. The diversity of the candidate gene functions highlights the complexity of glia-neuron interactions involved in the disease and demonstrates that more investigation need to be done in order to fully understand the multifactorial causes of neuronal dysfunctions or death in neurodegenerative diseases. Beside the impairment of *Drosophila* glial cells in a disease condition, I also investigated the role of glial cells in adult CNS homeostasis. Via the downregulation of a transcription factor common and specific to all adult glial cells, called *repo*, I uncovered new glial functions involved in general brain homeostasis. Finally, I emphasize the importance of non-autonomous effect of glial transcription in neuronal function and morphology, using the visual system as a model. The results suggest that *repo*-dependent glial function is constantly needed during adult life in order to maintain a functional nervous system.

Table of Content

List of Figures

List of Tables

Abbreviations

ALS	Amyotrophic Lateral Sclerosis
Alrm	Astrocytic leucine rich motif
Atro	*Drosophila Atrophin*
BBB	Blood Brain Barrier
caps	*Capricious*
ChIP	Chromatin ImmuniPrecipitation
CNS	Central Nervous system
kDa	Kilo Dalton
DLM	Dorso-longitudinal Muscle
DNA	Deoxyribonucleic acid
DRPLA	DentatoRubro Pallidoluysian Atrophy
DSHB	Drosophila Hybridoma Bank
ECL	Enhanced chemiluminescence
ERG	Electroretinogram
FBS	Fetal Bovine Serum
FDA	Federal Drug Agency
FDR	False Discovery Rate
FTD	FrontoTemporal Dementia
GAD	Gal4 Activating Domain
nGFP	Nuclear Green fluorescent Protein
GMC	Ganglion Mother Cell
GPCR	G Protein coupled Receptor
HD	Huntington's Disease
KD	Knock-Down
KO	Knock-Out
LD	Lipid Droplets
LHG	LexA-H-GAD
LOF	Loss-of-function
LRR	Leucine Rich Repeat
LTM	Long-Term Memory
MARCM	Mosaic Analysis with a Repressible Cell Marker
mfas	*Midline Fasciclin*
MR	Magnetic Resonance
NBs	Neuroblasts
Nls	Nuclear localization signal
NMJ	NeuroMuscular Junction

NSCs	Neural Stem Cells
PBS-T	Phosphate Buffer Saline- Twin20
PCR	Polymerase Chain Reaction
PFA	ParaFormaldehyde
PNS	Peripheral Nervous System
polyQ	polyglutamine
PrP	Prion Promoter
iPSCs	Induced-Pluripotent Stem Cells
Rh4	*Rhodopsin 4*
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
S2 cells	Schneider 2 cells
SAT	Secondary Axonal Tracts
SB	Sample Buffer
SBMA	Spinal and Bulbar Muscular Atrophy
SCAs	SpinoCerebellar Ataxias
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SOD	Super Oxyde Dismutase
TBS-T	Tris-Buffer Saline-Twin20
TRiP	Transgenic RNAi Ressource Project
trn	*Tartan*
Tsh	*TeaShirt*
TUNEL	Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling
UAS	Upstream Activating Domain
Ubi	Ubiquitous
UCP	Uncoupling Protein
UPL	Universal Probe Library
UTR	Untranslated Region
VDRC	Vienna Drosophila RNAi Center
VNC	Ventral Nerve Cord
v/v	Volume by volume
w/v	Weight by weight

Acknowledgments

There are so many people to acknowledge. This thesis is the result of four years of hard work and could not have been possible without the support of my family, my friends and my colleagues (/co-workers… ☺).

First, I would like to thank my supervisor, Manolis Fanto, who has believed in me from the beginning to the end. Always around to help me in the lab or up for none scientific related conversations. You have helped me gain confidence and made me progress in every aspect a scientist and a human need. A special honour to all previous and current lab members for all the interesting and non-interesting discussions we had over lunch-time, in the famous fly room and at the Friday pub. Raul, for sharing the first two years in the lab with me and being always smiling and in a good mood. Catarina, Olga, Adel for your help and vibrating debates and Nadine for her contribution to my project. I also wish to thank all the "fly" people from the MRC who shared the fly burden of being wrongly blamed when the CO_2 level in the building was down. I would also thank my second supervisor, Jean-Marc Gallo, always available to listen to me if I had issues or worries. Finally, I would like to thank Ataxia UK, which has given me the opportunity to do this PhD by funding the project.

Obviously my life was not only spent at work and my friends and family deserve a tremendous acknowledgement for their support and for making my life much better during my time in London. Merci beaucoup à mes parents et à mon frère for their constant support, and not only financial one ;) . A special thanks to Sandra, always here to listen to me when I was feeling bad and being the best to know what to say in those difficult moments. Also, thank you Nicolas and Olivier for maintaining my fitness over the years with very intense squash games. Thank you Emma for keeping our weekly emails happening during the four years almost without a miss, for visiting me regularly and for the welcome on my first days in this country. Of course I do not forget my bozos, also for their visits and their welcome during these years and for sharing "unfiltered" live thoughts. I keep the last line for my binôme, a special friend, who even at thousand miles away is the closest in my heart. You do not realize how much your presence has been helpful to maintain my happiness.

Chapter I:

Introduction

1.1 Glial cells

1.1.1 In mammals

There are five main types of glial cells in the mammalian central nervous system (CNS): the astrocytes, oligodendrocytes, microglia, ependymal cells and radial glia. The glial cells in the peripheral nervous system (PNS) is composed of mostly the Schwann cells, the satellite cells and the enteric cells (Rasband 2015). They are subdivided according to their morphology, function, origin and molecular compositions. The major difference from vertebrates to invertebrates is the presence of microglia, from mesodermal origin, which are the immune cells of the brain.

During development, gliogenesis follows temporally neurogenesis in mammalian CNS. The development of glial cells has been mostly characterized for astrocytes and oligodendrocytes, both of them originally coming from neuroepithelial cells. These cells will give rise to radial glial cells, which can generate either neurons or glial progenitors (Malatesta et al. 2000). These progenitors will then produce astrocytes or oligodendrocytes depending on a complex epigenetic/signalling regulation. Radial cells also generate ependymal cells. Also, there are temporal distinct waves of gliogenesis, producing oligodendrocytes in different regions of the brain while production and migration of astrocytes remains unclear because of the lack of tools differentiating radial glial cells, precursors of astrocytes and mature astrocytes (**Figure 1.1**) (Rowitch et al. 2010). The higher complexity in mammalian gliogenesis relates to a higher diversity of glial specialized functions and morphologies depending on their interactions with a greater diversity of neurons. For instance, astrocytes have been divided into two main categories, the fibrous astrocytes and protoplasmic astrocytes, residing in the white matter and grey matter respectively, thus having different functions.

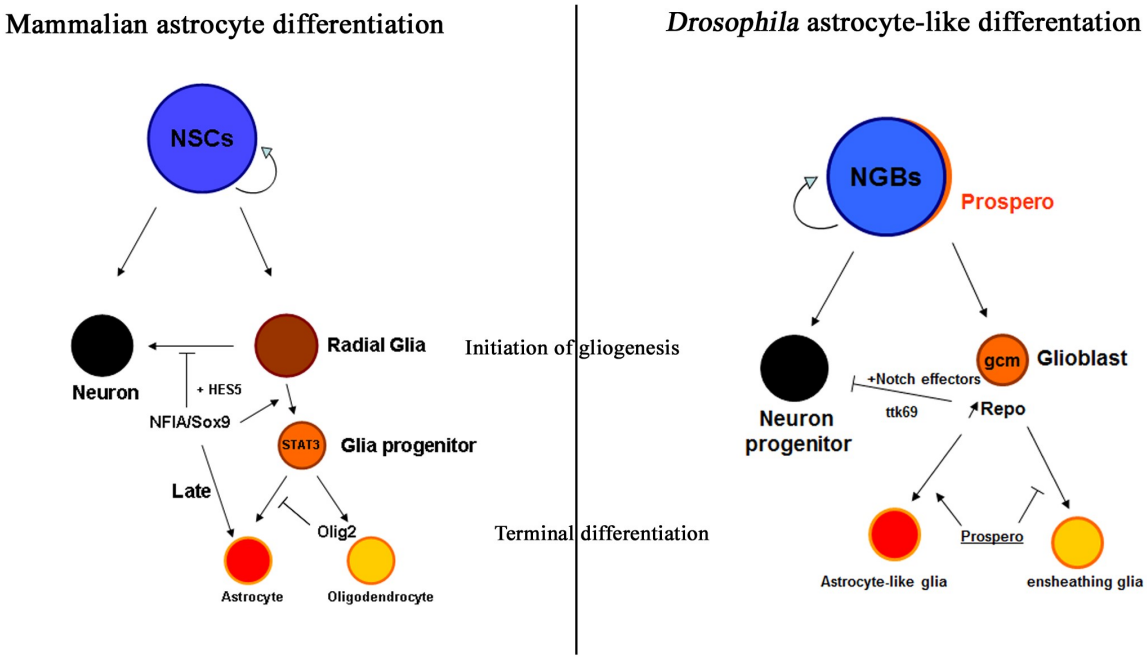


Figure 1.1: Molecular events leading to astrocyte differentiation in mammals and *Drosophila*. NSCs: Neural stem cells. NGBs: Neuroglioblasts.

Astrocytes have various physiological functions, one of them being an active member of the Blood-Brain-Barrier (BBB). Tight junctions are formed by the endothelial cells of the blood vessels and then, postnatally, astrocytes project endfeet surrounding the blood vessels and help maintain the function of the BBB. This system is called the neurovascular unit (**Figure 1.2**). Thanks to these direct interactions, astrocytes are proposed to regulate blood flow and also to take up glucose, convert it to lactate and release it to the neurons for local energy needs (Magistretti 2006).

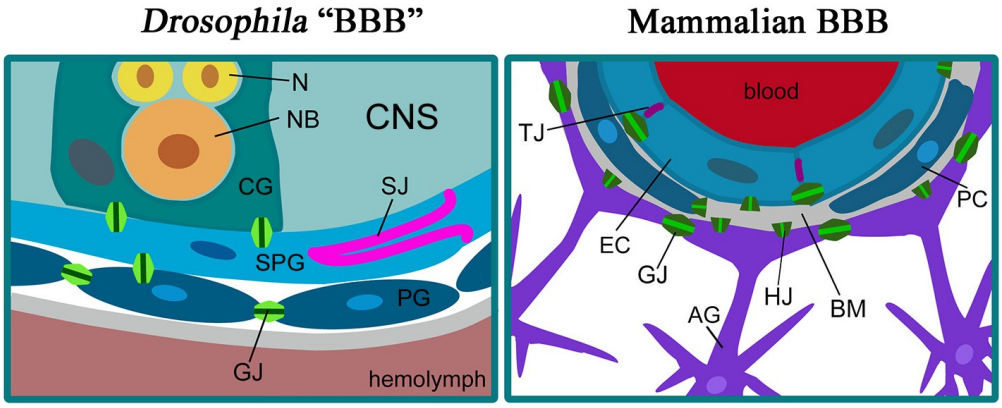


Figure 1.2: Comparison of the Blood-Brain-Barrier of mammals and *Drosophila*. N: Neuron, NB: Neuroblast, SJ: Septate junction, GJ: Gap junction, CG: Cortex glia, SPG: Subperineurial glia, PG: Perineurial glia, TJ: Tight junction, EC: Endothelial cell, AC: Astrocyte, PC: Pericyte, BM: Basal membrane, HJ: Hemichannels. Adapted from Limmer et al. 2014

The astrocytes are the most numerous glial cells in the CNS. They are highly ramified but do not overlap in territory (Bushong et al. 2002) (**Figure 1.3**). Besides contacting blood vessels, astrocytes are also extensively studied for their interactions with neurons at the synaptic level where in addition to clearing the synaptic cleft from neurotransmitters to avoid excitotoxicity, they also regulate their activity. A study suggests that one astrocyte could associate with 4 neurons, 10^5 synapses (300-600 dendrites) and 1-2 capillaries (Halassa et al. 2007). They regulate many components of the synapse: they buffer the K^+ concentration (Butt et al. 2006), the volume by water homeostasis (Nagelhus et al. 2004) and can compete with post-synaptic neurons for neurotransmitter modulating their activity (Pannasch et al. 2014). More than regulating the extracellular components of the synapses, astrocytes are also known to regulate the formation and the plasticity of synapses via the secretion of molecules such as thrombospondins (Risher et al. 2012). Interestingly, a very recent discovery has highlighted a potential bi-directional communication between neurons and astrocytes in the cerebellum, with the neurons also being able to control astrocyte properties via sonic hedgehog signalling (Farmer et al. 2016).

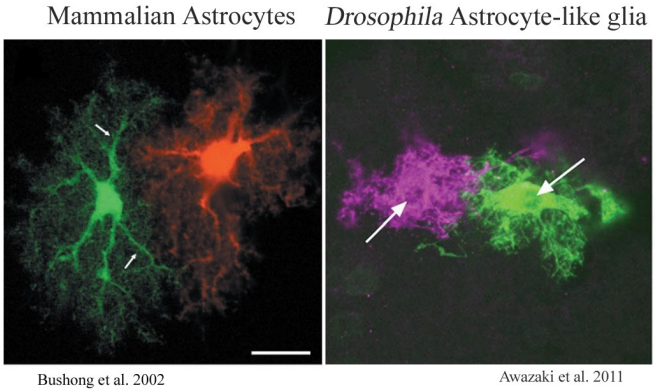


Figure 1.3: Both mammalian and *Drosophila* astrocytic projections do not overlap

Oligodendrocytes (CNS) and Schwann cells (PNS) are wrapping axons via myelin sheath. Contrary to the oligodendrocytes capable of wrapping many axons, Schwann cells wrap only one in the PNS, with one axon being wrapped by many Schwann cells in line with each other (Feltri et al. 2015). Both cell types actively recruit ion channels in a very controlled spaced manner along the axons, forming nodes of Ranvier. Together with the isolating properties of the myelin sheath, this clustering of ion channels increases the conduction velocity of axonal action potentials. These modifications of electric properties allow a reduced size of axons and use of energy to maintain ionic gradients (Howe et al. 1990). In mammals, axons can range from millimetre to meter (one meter for the sciatic nerve in human) and are isolated from the extracellular space except at the node of Ranvier. Thus, it was suggested that myelinating glia provide direct metabolic support to axons. It has been recently proposed that both cell types provide some metabolites such as cholesterol, lactate or glycogen (Hirrlinger et al. 2014).

The other most prominent glial cell type, even though from mesodermal origin, is microglia. This cell type is the immune cell of the brain, having highly ramified processes thought to cover a defined territory. Following an injury, microglia change shape, migrate to sites of injury and change their protein expression (Atallah et al. 2014). Once on the site of injury, they release cytokines which can have a dual role of either triggering cell death or survival and are also responsible for the removal of cell debris by phagocytosis (Cherry et al. 2014, Fernandes et al. 2014). However, beside this role of “immune cell”, it has recently been shown that a deficit in microglia could trigger behavioural deficits and impaired learning-dependent synaptic plasticity (Parkhurst et al. 2013), extending the role of microglia in the CNS. Evidence increasingly points toward not only a role in defence mechanisms but also in normal brain development and function (Schafer et al. 2012).

1.1.2 In *Drosophila*

In *Drosophila*, glial cells have not been studied as extensively as neurons. However, glial cells possess a wide range of functions that are being more and more investigated at the cellular and molecular levels. The development of glial cells has been very well characterized in *Drosophila*, from birth to mature positioning, especially in the embryonic Ventral Nerve Cord (VNC). Glial cells would account for roughly 10% of the cells in the *Drosophila* central nervous system (Edwards et al. 2010), which is much lower than the ratio in mammals of 1:1 (Azevedo et al. 2009, Herculano-Houzel 2014). There are two types of glial cells based on their origin. The midline glia derive from the mesectoderm and thus are distinct by their origins to the lateral glia, deriving from the neuroectoderm (Parker et al. 2006). At the molecular level, the difference in this origin is marked by the absence of the initial glial fate determinant *glial-cell-missing* (*gcm*) and the terminal glial fate determinant *reversed polarity* (*repo*) expressions, which are present in all neuroectodermal glial cells. The midline glia are present only during development and their known function is to regulate the cross of the midline by commissural axon tracts by the expression of guidance cues (Mitchell et al. 1996). The other glial cells come from different neural stem cells (NSCs), also called generally neuroblasts (NBs). Some NBs will only give rise to neurons, some to both neurons and glia (called neuroglioblasts, NGBs), and few only to glia, called glioblasts. During embryogenesis, NBs give rise to another NB and a daughter cell, called ganglion mother cell (GMC), which will divide once to produce neurons and/or glial cells. A second wave of neurogenesis during larval stage, after the remaining embryonic NBs will exit quiescence state, will give rise to 90% of the neurons in the adult CNS. Based on their positions and their lineage characteristics, mainly two types of NBs can be distinguished (in addition to VNC, mushroom body and optic lobe NBs). Type 1 NBs are similar to embryonic ones by giving rise to a GMC which will divide once more to produce two differentiated cells. The type 2 NBs divide asymmetrically and give rise to another NB and an transient cell called intermediate neural progenitor (INP), which will mature by a series of transcriptional changes and ultimately will produce a GMC and another mature INP (**Figure 1.1 and 1.4**) (Homem et al. 2012). The differentiation into glial cells is marked by the expression of *gcm*, triggered by *Prospero* which split

asymmetrically in the NGB from the former division into the future glial cell, to activate a stronger *gcm* expression, the initial glial cell fate determinant (**Figure 1.1**) (Freeman et al. 2001).

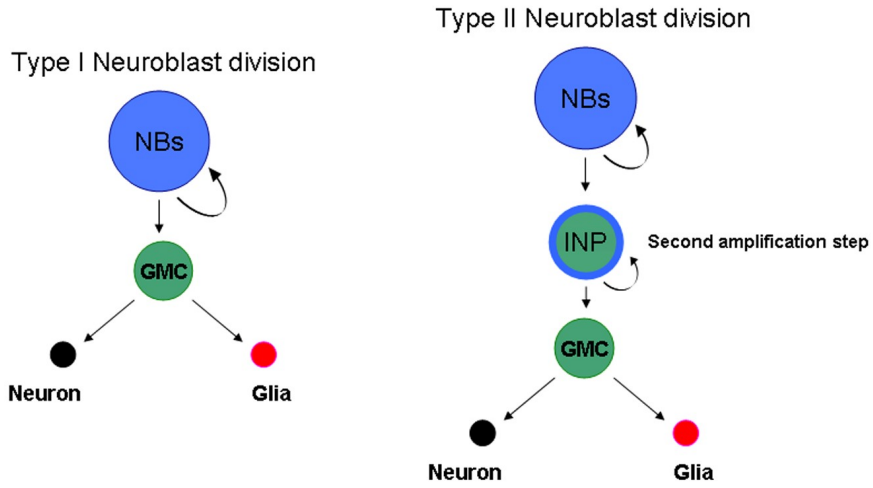


Figure 1.4: Type I and Type II neuroblasts division in *Drosophila* development

Different lineages of lateral glia have been extensively characterized by their positions and migrations in the VNC. Three main groups of glial cells are differentially produced. The first one is called surface associated glia and comprises subperineurial glia and channel glia. The second one is neuropil-associated glia, comprising longitudinal glia and nerve root glia and the third one is cortex-associated glia comprising only cell body glia (Edwards et al. 2010). There is also another type of non-neuronal cell present in the CNS, from mesodermal origin that can be considered as surface glia by their position, the embryonic

perineurial glia (Beckervordersandforth et al. 2008). They do not express *gcm* or *repo* in early development but do express *repo* later from larval stage and thus, can be considered as glial cells. However, the origin of the latter ones are supposed to be from quiescent neuroblast, questioning the similarities between the embryonic and post embryonic development of this outermost layer of the surface glia.

The subperineurial glia are considered to be the one forming the "Blood-Brain-Barrier" (BBB), isolating the brain from the K^+ rich hemolymph through tight septate junctions (**Figure 1.2**) (Limmer et al. 2014). The role of the perineurial glia has not been well investigated but a recent study has shown that these cells would be an important player in glial glycolysis for neuronal survival via the active import of trehalose from the hemolymph to the brain (Volkenhoff et al. 2015).

A second pool of glial cells is then produced during larval stages when the neuroblasts come out of their quiescent state (Sousa-Nunes et al. 2011). Then, in the adult stage, we can distinguish four types of CNS glial cells: the surface glia, the cortex glia, the neuropil glia and the "tracts" glia (not well defined yet) (Awasaki et al. 2008, Edwards et al. 2010). In the adult, beside the subperineurial glia, the surface glia comprise also the perineurial glia, which is the outermost layer of the BBB covered by an extracellular matrix called the neural lamella (Schirmeier et al. 2015). Both these layers actively participate in the filtration of molecules from the hemolymph to the CNS, in order to maintain a functional concentration of ions around the neurons and to provide them with energy metabolites (Limmer et al. 2014). The cortex glia are surrounded by the neuronal cell bodies and are able to wrap up around 20 neuronal cell bodies (Awasaki et al. 2008). By their location and being also directly in contact with the BBB glia, it has been suggested that they have a role in providing energy directly to the neurons (Freeman et al. 2006, Kis et al. 2015), which would mimic closely the role of astrocytes in mammalian BBB, highlighting functional similarities between vertebrates and invertebrates BBB (**Figure 1.2**). Furthermore, some of the cortex glia are also labelled by the *dEAAT1-Gal4* driver, constructed from the promoter of the only known *Drosophila* glutamate transporter (characterized in this thesis). It is thus possible that at least some of the cortex glia are able to regulate glutamate transports in the

cortex. In the visual system, the cortex glia are called satellite glia. Two different populations have already been isolated by the class of neurons they surround. However, it is not known yet whether such subdivisions apply elsewhere in the brain, although it is likely (Edwards et al. 2010). The neuropil glia have their cell bodies laying around the neuropil. They are composed of the ensheathing glia, surrounding the axonal bundles composing the neuropils and the astrocyte-like glia, sending projections in close proximity to the synaptic regions (Awasaki et al. 2008). The first ones help to isolate nerves and can also support neuronal survival by providing them with trophic support (Schirmeier et al. 2015). The astrocyte-like glia, named according to the similar star-shaped mammalian astrocytes, express neurotransmitter transporters and are thus important for neurotransmitter clearance and recycling (Agarwal et al. 2014, Chaturvedi et al. 2014, Stork et al. 2014). Similarly to mammalian astrocytes, astrocyte-like glia do not overlap in their projection domains (**Figure 1.3**) (Awasaki et al. 2011, Peco et al. 2016). They are also known to engulf neuronal debris during development but this function has not been proven yet in the adult (Tasdemir-Yilmaz et al. 2013). It is also possible that *Drosophila* astrocyte-like glia could regulate synaptic activity by controlling neurotransmitter and ion transports at the synaptic cleft. Indeed, it has been recently demonstrated that glial transcriptional activity via the transcription factor *repo* is needed for long-term memory (LTM) especially in astrocyte-like glia, which suggests an important role of *Drosophila* glial cells in the modulation of neuronal activity (Matsuno et al. 2015). Contrary to the mammalian CNS, *Drosophila* does not possess microglia. This role is taken over by different types of glial cells, among which the ensheathing glia (Doherty et al. 2009), the astrocyte-like glia (Tasdemir-Yilmaz et al. 2013, Hakim et al. 2014) and perhaps even the cortex glia, which express the engulfment receptor Draper (Doherty et al. 2009).

An increasing body of evidence suggests that *Drosophila* glial cells are very similar to their mammalian counterparts by developmental, morphological, functional and molecular criteria. However, contrary to the mammalian glia, the *Drosophila* ones have been extensively characterized, even to a single-cell resolution in the embryonic CNS thanks to the availability of a wide range of genetic tools and specific markers. Similarly to the mammalian glia, the *Drosophila* glia subtypes have not been clearly subdivided further

even though specific markers are already available. Further investigation of potential new subtypes of glia would provide a better insight into the functions of glial cells in a simpler organism, which however possesses very similar functional cells to maintain the homeostasis of its nervous system.

1.1.3 *repo* regulation in glial cells during development

repo is a transcription factor expressed specifically in *Drosophila* glial cells but does not have a clear ortholog in mammals, apart from DMBX1, the closest mammalian ortholog (with around 26% identity and 38% similarity). It contains a homeodomain that belongs to the paired (PRD)-like group. This homeodomain is closely related to the murine *Pax-3*, *C-elegans unc-4* and *Drosophila Aristaless*. It has been discovered in a screen looking at electroretinogram (ERG) recordings of mutants in *Drosophila*. Among 7000 lines tested, it was the only mutant that had a reversed polarity phenotype of the ERG. This mutant was a hypomorph allele of *repo*, called $repo^{1}$, which develops to the adult stage with abnormal glial localization in the optic lobe. Strong mutant alleles, called $repo^{2}$, $repo^{3}$ and $repo^{4}$ were embryonic lethal (Xiong et al. 1994). However, using the hypomorph allele, it was shown that the ERG was only reversed during few days post eclosure and then would be reduced to almost being absent after 14 days post eclosure. It would be mostly due to the neuronal apoptosis triggered by the disorganisation of the glia in the optic lobe, leading to the retina degeneration. Interestingly, split rhabdomeres could be observed, sometimes, in non-degenerating ommatidia (related to **Chapter V, section 5.2.5.3**) (Xiong et al. 1995). Following this discovery, *repo* was shown to be required for the nervous system development and for the differentiation and maintenance of glia functions during embryogenesis. However, it is necessary for the initial determination of glial cells (Halter et al. 1995). In the same study, it was demonstrated that it is expressed in all glial cells except the midline glia, which come from a different origin and do not express the initial glial cell fate determinant *gcm* either. *repo* being expressed only later during embryogenesis, *repo* mutant embryos display late developmental defects with disrupted axonal fasciculation and less glial cells. The migration of glial cells was also affected.

The expression of *repo* starts precisely at the stage 11 of embryogenesis under the direct transcriptional activation of *gcm*, with *repo* possessing 11 Gcm binding sites in its promoter region (Lee et al. 2005, Laneve et al. 2013). Then, it remains expressed in all glial cells until adult stage (see **Chapter V**) and (Soustelle et al. 2007, Matsuno et al. 2015). It has been demonstrated that *gcm* is the earliest glial cell fate determinant, directly switching cells to glial fate when expressed during early development, acting as a binary switch for glia versus neurons (Hosoya et al. 1995, Jones et al. 1995, Vincent et al. 1996). This activation of glial cell fate by *gcm* arises from the asymmetric localization of Prospero proteins during neuroglioblast division, which will trigger *gcm* expression to induce glia development (Freeman et al. 2001). After a complex regulation of Repo and Gcm protein levels via interlocked loops, the Repo protein will take over the regulation of its own expression and triggers *gcm* degradation and repression at the same time via competition with the *dCBP* acetylase at stage 14 (**Figure 1.5**) (Lee et al. 2005, Laneve et al. 2013, Flici et al. 2014). Lee et al. have further characterized *repo* transcriptional regulation by isolating a 4.2kb sequence upstream the *repo* start sites sufficient to trigger wild-type expression (related to **Chapter V, section 5.2.2.2**). However, different elements present in the regulatory sequence of *repo* would be responsible for its specific activity in subsets of glia. Moreover, according to the same study, other elements than *gcm* could be responsible of the *repo* activation in glia. Indeed, the presence of *gcm* in hemocytes during embryogenesis does not trigger the expression of *repo* in this cell lineage, from mesodermal origin (related to **Chapter V**, **section 5.2.1.2**).

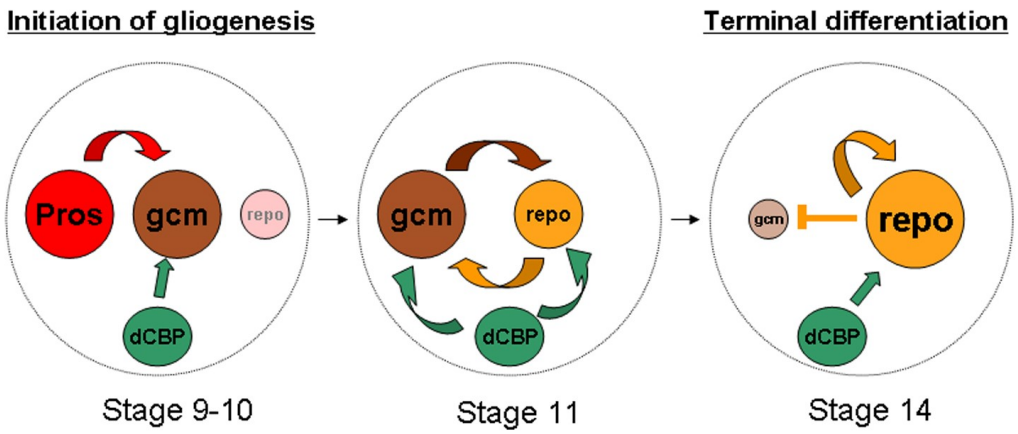


Figure 1.5: *gcm* and *repo* cross-regulation via a potential competition for dCBP acetylation. *gcm* expression is activated by Prospero, which initiate gliogenesis. *gcm* will in turn activates several genes such as *ttk69*, *pntp1* and *repo*. Repo would compete with Gcm for acetylation via dCBP interaction and later would block Gcm stabilization and trigger its degradation. After being activated by Gcm, repo expression would act via a positive feedback loop.

repo controls the terminal differentiation of glial cells. *gcm* directly activates several other transcription factors, which will cooperate to trigger different glial programs and fully differentiate subsets of glial cells. At the same time, this cooperation will also repress neuronal development, by synergic effect between *repo* and tramtrack69 (*ttk69)* (Yuasa 2003). Indeed, even though *repo* is expressed in all glial cells, other transcription factors, such as *ttk69* or *Pointed P1* (*pntp1)*, are expressed in specific subsets of glia. As an example, *loco*, a gene required for glial differentiation, morphogenesis and BBB formation (Granderath et al. 1999), is first triggered by *gcm* (Granderath et al. 2000) but its later

expression will become dependent on the cooperation between *repo* and *pntp1* (Yuasa 2003). It has also been shown that other specific glial genes are regulated by *repo*. In *repo* mutant embryos, terminal glial differentiation involving the expressions of the glutamate and taurine/aspartate transporters *dEAAT1/2* are abolished in glial cells (Soustelle et al. 2002). More recently, using chromatin immunoprecipitation (ChIP) in S2 cells (embryonic *Drosophila* cells), several potential *repo* direct target genes have been discovered. Different groups of genes have been highlighted, amongst which are specific glial genes, such as the only glutamine synthetase present in *Drosophila* (*Gs2*). This gene has been confirmed to be regulated by *repo* in *repo* null mutant embryo. However, even though it possesses *repo* binding sites in its promoter, a direct regulation has not been confirmed yet. Several other genes non-specific to glia have been found. Among them, several are involved in *wg/wnt* signalling (Kerr et al. 2014). In the same study, it has been confirmed that *repo* regulates the expression of *wg* in glial cells, and that glia-secreted *wg* at the larval neuromuscular junction (NMJ) regulates glutamate receptor clustering and synaptic physiology.

The function and regulation of *repo* during embryogenesis has already been well documented, however, its expression remains through all *Drosophila* stages and as a transcription factor, is expected to control other gene expressions. It is the case at the larval stage where its expression is necessary for the correct function of the NMJ. Moreover, it has recently been found that the expression of *repo* in adult glia is required for LTM (Matsuno et al. 2015). An adhesion molecule called Klingon (Klg) is expressed in both neurons and glia and its protein level increases upon LTM induction, which triggers an increase in *repo* expression as well. It is proposed that *repo* controls the activation of genes in glial cells that are necessary for neuronal activation involved in LTM via Klg-mediated communication between neurons and glia (Matsuno et al. 2015). However, this study is the only one investigating the role of *repo* in adult glial cells.

Even though *repo* does not have clear ortholog genes in mammals having a similar function, it seems that the logic of the molecular mechanisms regulating gliogenesis, at least in mice, are relatively similar, though to a higher level of complexity. In both cases, some transcription factors are used at the very early stages to trigger glial fate but later to

differentiate astrocytes from oligodendrocytes (ensheathing glia in *Drosophila*) as it is the case for *NFIA* (in mammals) and *Prospero* (in *Drosophila*) (**Figure 1.1**) (Deneen et al. 2006, Namihira et al. 2009, Kang et al. 2012, Peco et al. 2016). However, mammals are more complex, especially at the molecular level. In *Drosophil*a, a unique *gcm-repo* pathway controls general glial fate, with *repo* binding to different partners such as *ttk69* and *pntp1* to trigger glial subtype specification later on. In mammals, even though *Sox9* cooperates with *NFIA* to initiate glial differentiation, at least characterized for astrocytes and oligodendrocytes so far, at later stages, other transcription factors will take over the fate specification for oligodendrocytes. *Olig2* blocks *NFIA* function in oligodendrocyte while *Sox10* will cooperate with *Myrf* in the CNS to differentiate oligodendrocytes by activating myelinating genes for instance (Hornig et al. 2013) and with *Krox20* in the PNS Schwann cells. However, the role of *Prospero* in *Drosophila* astrogenesis has only been discovered very recently and more work would be required to investigate possible equivalent mechanisms for other glial lineages, similarly to what happens in mammals. Actually, similar complexity can also be observed between invertebrates and vertebrates in the redundancy of the use of one cell fate determinant transcription factor in different cell type. Similarly to *gcm* being expressed in both neuroectodermal glia and mesodermal hemocytes, triggering immune cell differentiation early in development of the *Drosophila* embryo, *Sox 10* is also expressed in melanocyte lineage in order to maintain melanocyte stem cell progenitors (Harris et al. 2013).

Overall, glial cells actively participate to the development and function of the brain at many different levels, from the maintenance of the BBB to the regulation of the synaptic activity. Being in such dependence to their trophic support and regulation, neurons are easily vulnerable when glial cells are impaired. It is now well established that glia have a major role in neurodegeneration, which can be either protective or harmful but could be still an interesting target for therapeutic intervention.

1.1.4 The role of glial cells in neurodegeneration

During neurodegeneration or after an injury, glial cells can change morphology, particularly observed for microglia, or change gene expression profile, such as an increase in *Gfap* expression level in astrocytes. However, it still remains controversial whether they are just reacting to neuronal impairment or if they are active players in the impairment.

In the case of astrocytes, this activation is called astrogliosis and is defined by morphological and molecular changes and is mostly characterized by an increase in *Gfap* expression (Burda et al. 2014) and extracellular matrix molecules forming a scar around the injury site (Yuan et al. 2013). This scar can have both positive and negative sides. The positive one is the limitation of the tissue damage due to inflammation by restricting the injured site (Sofroniew 2015). The negative side is that the formation of this scar has the potential of inhibiting axonal regeneration which is a big challenge to overcome after spinal cord injury for instance (Sharma et al. 2012). Beside the formation of the scar, cell-autonomous impairment of astrocytes due to the expression of mutated proteins such as polyglutamine expansion can lead to neurodegeneration by impairing glutamate transport for instance by changing the transcription profile of cells, either directly by the intrinsic function of the mutated protein or by sequestering other transcription factors or regulators in the aggregates (Custer et al. 2006). On top of that, the dynamic of polyglutamine protein expression in time and toxicity has not been assessed in glial cells and could be different from the one in neurons, which do not divide in the adult brain (apart from the subventricular zone). Indeed, the expression of *polyQ-Atro* in *Drosophila* adult glial cells for only 3 days does not reduce the lifespan (data not shown). Thus, the involvement of astrocytes in neurodegenerative diseases (as well as other glial cell types) has to be well defined for each of them in order to understand the role in the progression of the disease. A better understanding of their participation to the disease would help to design drugs either to promote some of their functions or to inhibit others.

The myelination of axons by oligodendrocytes or Schwann cells is also directly involved in some neurodegenerative diseases. De- or dysmyelination can result from different origin;

genetic, such as the Charcot-Marie-Tooth peripheral neuropathies or Pelizeaus-Merzbacher CNS hypomyelinating disease, or auto-immune, such as PNS demyelinating Guillain-Barre syndrome or CNS demyelinating multiple sclerosis. The major cause in these diseases is the loss of axonal support leading to axonal degeneration and permanent loss of function (Trapp et al. 1998, Lee et al. 2012).

Microglia, as immune cells, have a primary role in brain injury or disease via the secretion of cytokines responsible of inflammation, reactive oxygen and nitrogen species and proteolytic enzymes triggering cell death (Baburamani et al. 2014). However, similarly to astrocytes, they can also have a neuroprotective role, downregulating inflammation and promoting tissue repair. It is also suggested that microglia change in morphology and function with aging, resulting in a decline in their ability to repair and protect the CNS (Benarroch 2013). To add another layer of complexity of glial participation to neurodegenerative diseases, it has been suggested that inflammation plays an important role in neurodegeneration in multiple sclerosis (Derfuss et al. 2009). Moreover, a wrong expression of pro-inflammatory cytokines by microglia can trigger mitochondrial dysfunction in both glia and neurons (Lisak et al. 2009, Campbell et al. 2011). All these observations have attracted interest toward the use of drugs to modulate microglial activation for therapeutic purposes. However, the variety of their functions, either neuroprotective or neurodegenerative make the development of future drugs challenging by targeting specific functional states by suppressing deleterious effects and promoting protective ones (Correale 2014).

The importance of glial cells in the process of neurodegeneration is now taken into account in more and more studies and it has been already demonstrated that removing polyglutamine proteins specifically in glial cells can reduce the symptoms (Furrer et al. 2011) but also that expressing mutated proteins exclusively in glial cells is enough to trigger neurodegeneration (Besson et al. 2010, Nisoli et al. 2010, Lee et al. 2015).

1.2 *Drosophila* as a model for neurodegenerative diseases

Drosophila has been one of the first animal models to study genetics and has helped to describe a lot of conserved cellular mechanisms throughout its extensive use in laboratories. Numerous fly models for human diseases and especially neurodegenerative diseases have been backed up by a relatively recent study where it was estimated that 75% of genes related to a disease in human have orthologs in *Drosophila* (Bier 2005). Other "small" animal models have been developed in the attempt of deciphering molecular pathways involved in human diseases. Understanding the molecular mechanism behind cellular defects that lead to a certain phenotype (e.g. specific death of Purkinje cells in some spinocerebellar ataxias (SCAs) for instance) will increase the chances to find therapeutic treatments by isolating specific therapeutic targets or signalling pathways. The use of *Drosophila* has come as a good compromise between gene homology, fast life-cycle, reduced maintenance and costs and genetic tools available for molecular investigations.

1.2.1 Using *Drosophila* models for high-throughput screening

The use of *Drosophila* as a genetic model for human diseases has to be well characterized and its limitations have to be understood. Indeed, while most of the age-related neurodegenerative diseases appear in the mid-life in humans, developmental phenotypes are observed in flies. Many screens have been done in flies using the *GMR-Gal4* driver to find enhancer or suppressor of the famous "roughness of the eye" phenotype, associated with neurodegeneration (**Figure 1.6**) (Kazemi-Esfarjani et al. 2000). However, even though it is often ignored, this phenotype is the result of developmental defects of the eye, which does not form correctly and is mainly due to the dysfunction of the supportive non-neuronal cells. This questions the characterization of this phenotype as being "neurodegeneration". However, several screens carried out using this dogma have been partly successful, or at least have given indications toward potential molecular mechanisms underlying cellular defects. A fly model for the SpinoCerebellar Ataxia 1 (SCA1), an ataxic disease characterized by cerebellum degeneration and specific motor defects, has been used to

screen modifiers of this eye roughness (Fernandez-Funez et al. 2000). Even though in the same study Fernandez-Funez and his colleagues also characterize an age-related phenotype using a ventral-nerve-cord Gal4 driver specific to the adult, the *GMR-Gal4* driver was the one used for the screen and do not confirm their modifier effects using this other adult-specific driver. However, out of the genes discovered in the screen, two groups of them were known to be involved in the nuclear pore complex and RNA-processing, which are now thought to be two of the major pathways involved in the disease (Orr 2012) and this has been confirmed in mammalian cell culture (Irwin et al. 2005). Using the same paradigm of the eye roughness ➔ neurodegeneration, but this time on pre-validated compounds that went through a yeast assay of polyglutamine *huntingtin* (*htt*) aggregation, mammalian cell culture and then R6/2 mouse slices in culture, the use of *Drosophila* has confirmed the validity of some candidate drugs, as a first step of whole-animal model evaluation of FDA approved drugs (Zhang et al. 2005, Desai et al. 2006). However, those drugs were already known to be toxic in a long term use in humans, which is obviously a problem is this kind of diseases, showing the limitation of *Drosophila* for clinical evaluation of drug toxicity translatable to human. More success has been obtained using more developmental disease models such as for the study of the fragile X syndrome. Using mutant embryonic lethality of the *Fmr1* gene in *Drosophila* (ortholog of the *FXR1* in human responsible for the disease), a screen of 2000 FDA approved compounds has highlighted the importance of GABA signalling in the disease (Chang et al. 2008). Indeed, it has been shown in a mouse model of the disease that a GABA receptor antagonist rescues some of the symptoms (Selby et al. 2007).

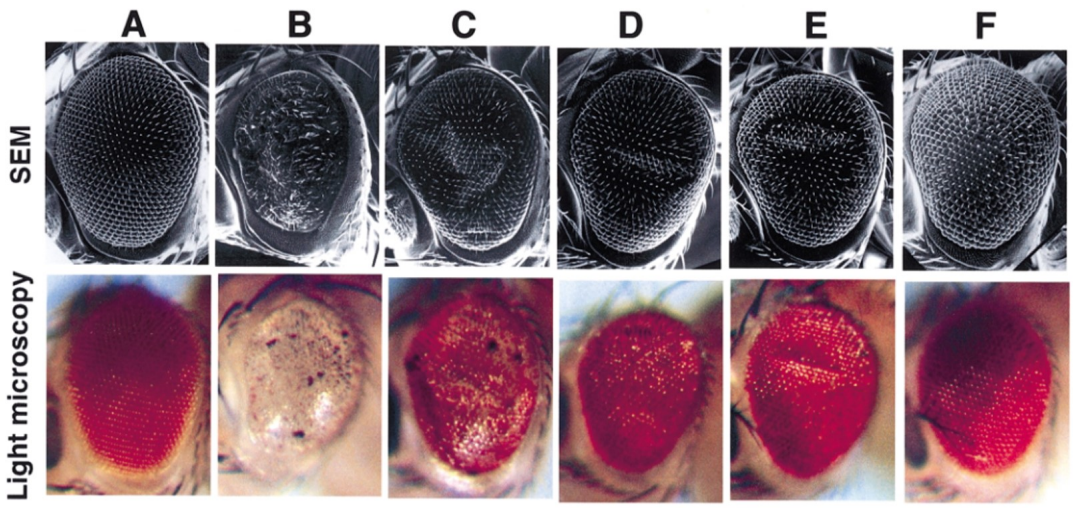


Figure 1.6: Different example of modulation of the roughness of the eye using GMR-Gal4 driver to express a poly Q repeat expansion with different toxicity modifiers. A is wild-type and B UAS-127Q. Adapted from Kazemi-Esfarjani and Benzer et al. 2000.

More recently, adult-specific screens have started to emerge in order to model more reliably age-related phenotypes and thus, investigate more accurately adult specific molecular defects effective with ageing. Using the strategy of drug-inducible system to allow specific expression in a time-controlled manner, a screen using a *Drosophila* SpinoCerebellar Ataxia 7 (SCA7) model has been done in the adult fly neurons and has identified a drug, sodium butyrate, a histone deacetylase inhibitor, as a potential candidate drug that improves survival in adult fly (Latouche et al. 2007), which seems appropriate given that the SCA7 gene, *ataxin-7*, encodes for a protein known to be involved in histone acetylation. In the same study, the results were confirmed using rat cortex cell culture. Sodium butyrate has also been shown to have a positive effect on the DentatoRubro-PallidoLuysian Atrophy

(DRPLA), a polyglutamine disease caused by the expansion of the glutamine stretch in the *Atrophin-1* gene, being a transcription co-repressor (see **1.4.2** section). Indeed, common mechanisms have been found between polyglutamine (polyQ) diseases (see **1.3** section). It is actually using *Drosophila* that for the first time it was shown in vivo that the expression of polyQ alone was intrinsically toxic and caused neurodegeneration (Marsh et al. 2000).

1.2.2 The genetic power of *Drosophila* for molecular dissection of disease mechanisms

Drosophila models are not only used for screening genes or pharmacological compounds on big scales. The genetic tools available to target the expression of transgenes in a cell-specific and time controlled manner allow a deeper investigation of molecular mechanisms involved for each disease modelled in *Drosophila*. The case of the mitochondrial defects involving *parkin* and *Pink1* in the familial cases of Parkinson's disease (PD) is the perfect example as it has been first discovered in *Drosophila* models phenocopying human symptoms when *Drosophila parkin* and *Pink1* were mutated (Park et al. 2009, Muñoz-Soriano et al. 2011). The similarities in the molecular pathways between mammals and invertebrates involved in this disease allow a better understanding of the pathways potentially affected in the disease by studying *Drosophila* ortholog genes. Another conserved pathway between species is the autophagy pathway, increasingly found to be defective in many neurodegenerative disorders. It is the case for the DRPLA where a fly model has been used to investigate the molecular mechanisms involved in the defects, also observed in a DRPLA mouse model (reviewed in the **1.4** section) or for the Vici syndrome where *epg5* has also been shown to be involved in autophagy, observed in a fly model and human brain tissue of patients with this disease (Cullup et al. 2013, Byrne et al. 2016).

The use of the fly as a model is not even limited to genes having orthologs between mammals and invertebrates. The example of the investigation of the ALS-related gene C9orf72 is interesting since this gene does not have an equivalent in *Drosophila*. However, the use of several different models of the disease by overexpression of G4C2 repeats in different transgene environments has allowed the discovery of a major pathway potentially

directly involved in the disease. Indeed, several independent studies have discovered through specific screenings that the nuclear-cytoplasmic shuttle was affected by this repeat and confirmed the data in iPSCs from patients and post-mortem human brain tissue, beside the publication of a similar independent screen carried out in yeast demonstrating similar results (Freibaum et al. 2015, Jovičić et al. 2015, Zhang et al. 2015). These major discoveries confirmed in human patient samples were made using fly models expressing an exogenous gene not even present in *Drosophila*, highlighting the power of this model for understanding conserved cellular and molecular pathways involved in various diseases, especially neurological disorders reviewed in this section.

1.2.3 Improving the understanding in the glia-neuron communications impaired in neurodegenerative diseases

In most cases, the models are designed to study the effects of mutant genes on neurons and very few studies have come out about the importance of glial cells in neurodegenerative mechanisms. Two recent studies exemplified the importance of glial cells in CNS homeostasis in *Drosophila* via neuronal mitochondrial dysfunction or energy-dependent functions. Indeed, one study has demonstrated that neuronal mitochondrial dysfunction accompanied by generation of reactive oxygen species (ROS) often encountered in neurodegenerative disease triggers accumulation of lipid droplets (LD) in glial cells. Reducing LD accumulation in glial cells was able to lower ROS significantly and delays the onset of neurodegeneration, also observed in an *Ndufs4* mutant mouse having similar defects showing a conserved phenomenon in the interaction between glia and neurons (Liu et al. 2015). Another interesting study has shown that a single mutated gene, in this case *Drosophila htt*, could have different effects depending on its localized expression. It was shown that the energy-dependent functions of glial cells challenged by the expression of polyQ *htt* could be rescued by the modulation of the expression levels of the mitochondrial uncoupling proteins (UCPs) by increasing the glial glucose uptake, but failed to do so in neurons. On the other hand, the expression of the superoxide dismutase (SOD) and catalase to reduce the oxidative stress in neurons improved the neuronal toxicity triggered by polyQ *htt* but failed to do so in glial cells, demonstrating differences in mechanisms affected

between glia and neurons (Besson et al. 2010). These studies suggest that glial cells can be directly involved in neurodegeneration, independently of the neuronal expression of the mutated protein and highlight the differences in the mechanisms that can lead to the cellular defects in these two cell types. The emergence of glial cells as an important component of neurodegeneration has started to be also investigated in mice models with the example of the expression of *ataxin-7* (SCA7) in Bergman glia leading to Purkinje cells degeneration via the impairment of glutamate transport (Custer et al. 2006, Furrer et al. 2011).

Overall, the use of *Drosophila* as a model for studying human diseases has proven to be a rapid and effective way to decipher molecular pathways involved specifically in each disease, with either common defects, such as autophagy, or different ones, such as energetic functions for glia and oxidative stress in neurons for HD. Thus, it allows the dissections of conserved molecular mechanisms that would not be possible in mammalian models. Furthermore, it is also extensively used for high throughput screening of genes or pharmacological compounds to narrow down the number of potential positive candidates to evaluate in mice, which has a much higher cost.

1.3 Polyglutamine (PolyQ) diseases

At the beginning of the 90's, a new class of neurodegenerative diseases started to be characterized. Spinal and bulbar muscular atrophy (SBMA) was the first shown as being the result of an expansion of unstable trinucleotide repeats, CAG, present inside the androgen receptor gene (La Spada et al. 1991). The CAG triplet codes for the amino acid glutamine. Several neurodegenerative diseases have been characterized with this CAG repeat abnormal expansion: Huntington's disease (HD), SBMA, DentatoRubro-PallidoLuysian Atrophy (DRPLA) and 7 SpinoCerebellar Ataxias (SCA1, SCA2, SCA3, SCA6, SCA7, SCA12 and SCA17) (Margolis et al. 1993, Rubinsztein et al. 1993, Koide et al. 1994, Orr et al. 2007). On average, the size of the CAG repeat ranges from 5 to 35 and expands from 40 to 100 in disease condition, leading to the formation of big aggregates at

the cellular level. The expansion would occur in the gamete during cell division. The transcriptional machinery would abnormally generates extra number of CAG repeats due to the misreading of a long repeated unstable DNA sequence. All these neurological disorders are dominantly inherited. They are progressive by increasing neuronal dysfunction and mostly striking in midlife with an average age of onset of 40 years old. Another common feature is the negative correlation between the size of the repeats and the age of onset and the severity of the disease (**Figure 1.7**) (Zoghbi et al. 2000). However, the speed of the progression does not seem to correlate with the length of CAG repeats (Hasegawa et al. 2010).

The specificity of neuronal populations affected in each disease remains to be understood. Indeed, each of the proteins involved are widely expressed in the brain but also in other tissues. However, even though the SCAs are mostly affecting the cerebellum, each of these diseases has special features in terms of brain regions affected or neuron/fibre loss, thus leading to different symptoms. The most affected regions/cell populations in this group of diseases are the cerebellar Purkinje cells, dentate nucleus, brain stem and basal ganglia that can be shared between some, or the striatum, which is more pronounced in the HD for instance. Most of them if not all, lead to motor dysfunction, though with different characteristics such as chorea, dystonia, myoclonus, rigidity, parkinsonism and generally ataxia, tremor and dysarthria for the SCAs and DRPLA. However, other symptoms can appear such as dementia, seizures, heart failures and memory deficits (Orr et al. 2007).

Disease	Gene locus	Gene product	Normal CAG(n)	Expanded CAG(n)	Protein localization	Special features	Brain regions most affected
SBMA	Xq11-12	Androgen receptor	9–36	38–62	Nuclear and cytoplasmic		Anterior horn and bulbar neurons, dorsal root ganglia
HD	4p16.3	Huntingtin	6–34	36–121	Cytoplasmic	Intermediate alleles: 29–35	Striatum, cerebral cortex
SCA1	6p22-23	Ataxin-1	6–44	39–82	Nuclear in neurons	Normal alleles >21 repeats interrupted with 1–4 CAT units	Cerebellar Purkinje cells, dentate nucleus; brainstem
SCA2	12q23-24	Ataxin-2	15–31	36–63	Cytoplasmic	Normal alleles interrupted with 1–2 CAA units	Cerebellar Purkinje cells, brain stem, fronto-temporal lobes
SCA3	14q24.3-31	Ataxin-3	12–41	62–84	Cytoplasmic		Cerebellar dentate neurons, basal ganglia, brain stem, spinal cord
SCA6	19p13	CACNA1A	4–18	21–33	Cell membrane		Cerebellar Purkinje cells, dentate nucleus, inferior olive
SCA7	3p12-p21.1	Ataxin-7	4–35	37–306	Nuclear	Intermediate alleles: 28–35	Cerebellum, brain stem, macula, visual cortex
DRPLA	12q	Atrophin-1	6–36	49–84	Cytoplasmic		Cerebellum, cerebral cortex, basal ganglia, Luys body

Figure 1.7: Different characteristics of some of the polyglutamine diseases. Adapted from Zoghbi et al. 2000.

Several of these diseases have different models, from cells to mice, but also *Drosophila*. Some therapeutical strategies attempt at using CAG specific antisense oligonucleotides to knock-down or splice the CAG expanded exon but the fact that the non-affected allele also carries CAG repeats, albeit shorter, makes this strategy difficult to apply (Glorioso et al. 2011, Arechavala-Gomeza et al. 2014). Thus, animal models can help to understand the specific biology behind each of these disorders in order to find either specific or common pathways affected by the polyglutamine proteins. Understanding why only specific neuronal populations are affected and what the impaired cellular mechanisms behind it are, would help finding new therapeutic interventions for these neurodegenerative diseases, which unfortunately are so far void of any therapeutic treatments.

1.4 DRPLA

1.4.1 Characterization of the disease in patients

Following the discoveries of triplet repeat expansions in 1991 being responsible for the SBMA, several attempts to identify new genes containing triplet repeats responsible of uncharacterized diseases were done. One study led to the discovery of 14 novel human genes of which 8 were sequenced and analysed for their repeats (Li et al. 1993). In this study, they found one clone called CTG-37, which appeared later on to be the gene coding for Atrophin-1, responsible for the DRPLA (Koide et al. 1994, Nagafuchi et al. 1994). These two studies confirmed that the repeat length negatively correlates with the age of onset and the severity of the symptoms in DRPLA, comparable to other polyglutamine disorders. Also, Nagafuchi et al. found that there was an intermediate repeat length in the Japanese population studied, which could predispose certain populations to further expansion over generation leading to DRPLA. These intermediate lengths have already been reported for other diseases such as myotonic dystrophy (DM), fragile X syndrome and HD. The first cases in Europe were reported in United Kingdom and Malta (Warner et al. 1994). Though, it has to be mentioned that having very similar symptoms to HD, misdiagnoses were common. In the same study, more detailed later on (Komure et al. 1995), it was also reported that the increase in repeat length over generation was more prominent when coming from the father than from the mother, probably due to the instability of the repeat during spermatogenesis rather than in oogenesis. However, a single-cell analysis has demonstrated that the expansion can be very heterogeneous within tissues. For instance, the DRPLA allele in the cerebellum shows smaller expansion than in the molecular layer and white matter. Also, repeat expansions are more prominent in the Purkinje cells than in the granule layer but still less than in the glial cells, especially in the late onset patients (Hashida et al. 2001). These findings demonstrate that there are differences in length within neuronal subgroups and that number of cell division cycles is not only the determinant for this mosaicism.

The brain regions affected in DRPLA are rather specific and different from the other polyglutamine diseases. Indeed, while most of the SCAs affect the Cerebellum and the brain stem and for HD the striatum, in DRPLA, it is mostly the dentate nucleus, the red nucleus and the globus pallidus. As a result, it is characterized by various clinical features including ataxia, chorea, epilepsy, myoclonus, cognitive impairment and psychiatric symptoms. Epilepsy and myoclonus are frequently observed in early-onset patients whereas the other symptoms appear at later onset. The age of onset varies from 0 to 72 years old and the age of death from 18 to 80, with an average of 49 y/o (Hasegawa et al. 2010). The analysis of the brains regions affected by neurodegeneration was helped by the case of a father and his son who went through magnetic resonance (MR) examination prior to death and in whom post-mortem examination of the brains were obtained (Sunami et al. 2010). MR imaging showed atrophy of the cerebellum and the brain stem, lesions of the cerebral white matter and brain stem, progressive cerebral atrophy, which were more marked in the patient with the juvenile-onset DRPLA. The lesions observed in the white matter and brain stem reflect loss of myelinated fibers. Also, cerebral atrophy mainly corresponds to atrophy of the neuropil. Neuronal loss was observed along with astrocytosis in the dentate nuclei, subthalamus and external segment of the globus pallidus. Neurons in the pontine nuclei and in the cortical layers were shrunken, with phosphorylated neurofilaments.

The *Atrophin-1* gene codes for a protein identified as 190kDa in normal human (with 25-30 glutamine repeats on average) but shift up on Western blot in DRPLA patients (with 60-70 glutamine repeats on average) (Yazawa et al. 1995). Accumulation of aggregates in neuronal cytoplasm of DRPLA patient brains was reported (Yazawa et al. 1995) though it is now known that aggregates are formed in both cytoplasm and nucleus, interestingly with different dynamics (Hinz et al. 2011).

It has been demonstrated, by isolating DRPLA protein complex, that the cytoplasmic inclusions and the nuclear membrane were aberrantly phosphorylated in neurons, suggesting that the nuclear membrane could be another pathological feature of degenerating neurons in DRPLA (Yazawa 2000). However, in order to study the molecular mechanisms that lead to these defects in humans, different animal models have been generated.

1.4.2 DRPLA mouse models

Three mouse models have been generated so far, mostly with different CAG repeat number, going from 65 glutamine repeats to 129 (in the range of human disease length, compare to around 10-20 for non-disease length), cloned in the human *Atrophin-1* (Sato et al. 1999, Schilling et al. 1999, Ying et al. 2005, Sakai 2006). All these models have different characteristics from age of onset to type of symptoms. For instance, the mouse model generated with 65 repeats has a lifespan of about 12 months but starts to develop severe tremors and impaired gait by 2-4 months (Schilling et al. 2001). This model also displays nuclear aggregation of a 120kDa fragment, present in human tissues as well. The symptoms resemble closely the phenotype of human DRPLA patients having ataxia, tremors, abnormal movements and seizures. Interestingly, this model develops aggressive behaviour, similarly observed in patients, which is often associated with abnormalities in the serotonergic system. However, the levels of serotonin and dopamine (related to motor activity) were normal, suggesting that the defects in these pathways could come from the receptors of these neurotransmitters or additional unrelated pathways involved in aggression.

Another mouse model, this time generated by cloning human *Atrophin-1* gene in embryonic stem cells, possesses 76 to 78 repeats. This model has been extensively used to study intergenerational CAG repeats instabilities. Indeed, similarly to humans, this mouse model displays an increase in the CAG expansions specifically via male transmission (Sato et al. 1999). This model, unlike the others, could be used to investigate the molecular mechanisms of instabilities of CAG repeats and highlights the complexity of the genomic sequence surrounding the gene enabling such instability. One such expansion, containing 129 CAG repeats, shows a strong neurological phenotype similar to the juvenile form of DRPLA (cerebellar ataxia, myoclonus and epilepsy, and die by 16 weeks of age). Interestingly, no obvious neuronal loss could be observed in any brain regions (Sakai 2006). This model has been used to analyse more in details the morphology of neurons and found changes in dendritic spine morphology and number and shrinkage of Purkinje cells dendrites (Sato et al. 2008). Using different sizes of expansion, 76, 96, 113 and 129Q, the

same group has characterized the evolution of the changes of behaviour and gene expression over time in a length dependent manner (Suzuki et al. 2012).

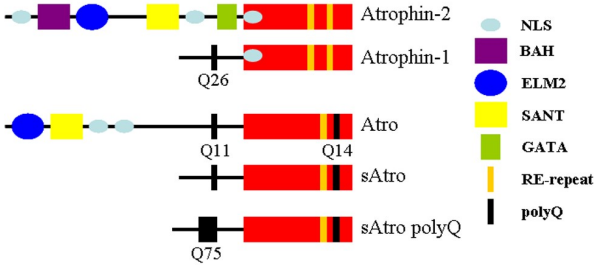
The generation of these models has allowed the investigation of molecular mechanisms underlying cellular defects due to the polyQ expansion. Indeed, one of the first discoveries of such abnormalities at the molecular level was the fact that polyglutamine proteins are able to interact more strongly to other proteins containing short polyglutamine tracts, as it is the case for the transcriptional co-activator CREB binding protein, CBP (Nucifora Jr 2001). It was found in both HD and DRPLA mice models that CBP was depleted from its normal nuclear location, interfering with CBP-activated gene transcription. This finding was further strengthened by another study where they found similar genes with abnormal expression levels, common to HD, DRPLA but also to SBMA and SCA7 mice models (Luthi-Carter et al. 2002). These results show that even if the proteins affected are different between all the polyglutamine disorders, they still share some common molecular defects independent of protein context. Also, the large alterations of gene expression in the brain due to retaining of transcription factors will certainly lead to the disruption of many different downstream molecular pathways, increasing the complexity of defects at the cellular level. Actually, using a DRPLA mouse model with 118 glutamine repeats, it has been shown that the use of sodium butyrate, a histone deacetylase inhibitor, ameliorates neurodegenerative phenotypes by modifying the histone acetylation profile (Ying et al. 2005). These mice also had strong reduction of their lifespan and dramatic impairment of their motor capacity. These results further confirmed that transcriptional changes play an important role in the pathogenesis of DRPLA and suggest that the reversion of the transcription repression could be an approach for therapeutic intervention.

Overall, using different models, it is quite clear that the polyQ stretch expansion in the *Atrophin-1* gene leads to changes in the transcription profile of the neurons and could be an important feature of this pathophysiology. The symptoms were triggered at different time points with different lifespans according to the model used but no obvious neuronal loss could be observed in any case.

However, most groups have disregarded the participation of glial cells in DRPLA. One group has investigated the potential implication of oligodendrocytes since it was reported in human DRPLA brain tissue that there was a reduced number of glial cells in the lesions and an involvement of oligodendrocytes in the nuclear inclusion formation. They suggested that glial cells could actually be involved in the pathogenesis (Yamada et al. 2002). Indeed, following the discovery of white matter damage and that polyQ Atrophin-1 immobilizes GAPDH, glycolysis and related pathways were investigated. It was concluded that along with astrocytosis present in the white matter damaged regions, CAG triplet expansion caused mismetabolism of polysaccharides contributing to the formation of the lesions in DRPLA (Shiozawa et al. 2003). In our lab, using the 65Q mouse model, it has been established that both transient astrocyte activation in the corpus callosum, and chronic astrocytosis in the internal capsule are displayed with the progression of the disease (unpublished). All these studies suggest that beside the clear direct implication of neurons in the disease, glial cells could at least participate in the progression of DRPLA.

1.4.3 Using *Drosophila* models to study DRPLA

The in vivo function of *Atrophin-1* in mammals was unknown when it was discovered that there were transcriptional changes in DRPLA. The use of *Drosophila* as a model for the genetic dissection of its function helped in the understanding of its role in the pathogenesis DRPLA. The Drosophila homolog, called *Grunge* (*Gug*) but later renamed *Atro* (used in this thesis), was discovered and characterized in 2002 (Schematic below and **Figure 4.1A**) (Zhang et al. 2002). It was shown to be a transcriptional corepressor in multiple developmental processes and the authors proposed that a deregulation of transcription could contribute to the pathogenesis of neurodegeneration. These results support the data from the mouse models and point toward a more direct implication of Atrophin proteins in the transcriptional deregulation rather than just sequestrating other transcription factors in inclusion bodies.



Schematic from **Figure 4.1** comparing mammalian *Atrophins* to *Drosophila Atro*

Furthermore, *Drosophila Atro* has been demonstrated to physically interact with *fat* to control planar cell polarity during development (Fanto 2003). This functional analysis during development led to the later discovery that in a DRPLA *Drosophila* model, polyglutamine expansion in the *Drosophila Atro* protein triggers neurodegeneration by downregulating *fat* in adult flies (Napoletano et al. 2011). Furthermore, it was shown that the defects were not due to the developmental function of the *fat*/*Hippo* signalling on regulating growth, a known pathway well characterized for tumour growth but were rather due to autophagic deregulation, affecting cell homeostasis. The characterization of the autophagic defects was done using the same model and demonstrated that the expansion of the polyglutamine stretch in *Atrophin* proteins blocks the autophagic digestion at the lysosomal level and that contrary to other autophagic defects, neurodegeneration was not rescued by inducing more autophagy (Nisoli et al. 2010). This model has given a better insight into the potential cellular mechanisms affected by deregulation of transcription triggered by polyglutamine *Atrophin*. The discovery that *polyQ Atro* downregulates *fat* and triggers autophagic defects in neurons has now been confirmed in a mouse model, similar to the 65Q one develop by Schilling et al. (Baron et al. submitted). The similarities between the invertebrate and vertebrate models in the cellular pathways involved DRPLA allow a better confidence in using *Drosophila* as a model for further studies of this neuropathological disorder.

1.5 Aim

To provide insights into the role of "diseased" glia during neurodegeneration, the aim is first to characterize the importance of each glial cell population using the expression of a toxic protein responsible for a rare neurodegenerative disease called DRPLA. Then, in order to investigate the interactions occurring during the progression of the disease, a new fly model is generated in which glial cells are specifically impaired and neuronal gene expression is modulated. The screening of neuronal genes involved in the modulation of the phenotypes given by the impaired glia will shed lights on potential new communications between neurons and glia involved in the progression of the disease. Such genes can open up avenues to finding new therapeutic targets that could delay neuronal death and maintain a functional nervous system longer, which would improve patient disease conditions. On the other hand, the general understanding of glial functions in healthy condition can improve our knowledge on potential new mechanisms regulated by glia, thus, understanding better their interactions with neurons but also with themselves. The second part of this thesis aims at unrevealing glial functions and regulatory pathways in the adult *Drosophila* nervous system.

Chapter II:

Materials & Methods

2.1 Genetics

2.1.1 *Drosophila* stocks and husbandry

Flies were maintained at either 18°C or room temperature on standard cornmeal agar medium (0.8% w/v agar, 2% w/v cornmeal, 8% w/v glucose, 5% w/v Brewer's yeast, 1.5% v/v ethanol, 0.22% v/v methyl- 4-hydroxybenzoate, 0.38% v/v propionic acid). The w^{1118} (w^{-}) was used as control (unless mentioned otherwise) and for RNAi TRiP lines, an *mCherry*-RNAi in the same background was used. Genetic manipulations, including balancers and phenotypic markers are described in Lindsley & Zimm (1992) and Flybase (www.flybase.org). The RNAi lines used for the screen or throughout the study are from either the VDRC (Vienna Drosophila RNAi Centre) collection (KK and GD lines) or from the Bloomington Stock Centre (TRiP lines).

***Drosophila* stocks used**: All the following lines were obtained from the Bloomington collection: w^{1118}, *Repo-Gal4*, *dEAAT1-Gal4* (II and III chromosomes), *NP2222-Gal4*, *Gliotactin-Gal4*, *MZ0709-Gal4*, *Moody-Gal4*, *UAS-CD8-GFP*, *UAS-nls-GFP*, *LOT-mCherry*, *GMR-Gal4*, *Elav-Gal4*, *Ubi-Gal80^{ts}* (II and III chromosomes), *mfas-RNAi* (TRiP 59205), *caps-RNAi* (28020), *trn-RNAi* (28525 and 50520) and *UAS-dEAAT1* (8202). The *mfas-RNAi* (103621 KK and 37888 GD), *caps-RNAi* (3046 GD and 27097 GD) were obtained from the VDRC collection in addition to all the RNAi lines used for the screen.

Alrm-Gal4 (II and III chromosome) and *UAS-Gat* were kindly given by M. Freeman. *Elav-Gal80* and *Tsh-Gal80* were kindly provided by R. Sousa-Nunez. *Repo-Gal80*, *UAS-Repo#9* (*myc-repo*), *UAS-Repo#14* (*myc-repoΔAD302*), *UAS-Repo*, *UAS-Repo* RNAi 1 and 2 (GD 10424 and TRiP 28339 respectively), *Repo-nGFP* and *Repo-LexA* were given by A. Giangrande. *UAS-miR-1* and *miR-1 KO* were given by E. Lai. *UAS-Atro75QN* was generated in a previous study (Nisoli et al. 2010). *UAS-Gs2* was given by R.W. Ordway, *UAS-loco* was given by U. Gaul.

The *dEAAT1-LexA::GADs*, *dEAAT1-LexA::VP16*, *LOT-wt-Atro* and *LOT-polyQ-Atro* were generated in this study.

2.1.2 Transgenic flies

Purified plasmids containing the transgenes of interest and the mini-white "w^+" marker gene were sent to inject to Fly-Facility company for *LOT-sAtro* wild-type or polyQ and for *dEAAT-LexA*. All transgenic flies that developed to adult were crossed to w^{1118} (w^-) flies. The eye color (w^+) of the progeny was used to identify the transgenic flies that had inserted the plasmid containing the mini-white gene and thus, the transgene of interest. Males with eye-color were then crossed to w^- balancer stocks on the second (If/CyO) and on the third chromosomes (TM3/TM6) to identify the location of the transgene insertion.

2.2 Behavioural assays

2.2.1 Lifespan

Crosses were maintained at 18°C during the developmental stages of the progeny. Newly eclosed adult flies were collected within 5 days at 18°C. 60 flies were selected per genotype, 30 females and 30 males, equally distributed within 3 vials. Lifespan is done in a controlled environment at 29°C and 60% humidity. Using CO_2 to asleep the alive flies, dead and alive flies are counted and transferred into new fresh vials three times per week. Data are analysed using GraphPad Prism software. Statistical analysis was done using the log rank test of the Kaplan and Meier method. The Chi square (χi^2) value was used as a measure of strength to compare the phenotypes as it is directly proportional to the difference between two curves and proportional to the P-value. χi^2 also takes into account the number of flies assessed. Thus, this value can be compared between different lifespan only when similar number of flies was used. The median survival of each curve will also be used to compare the curves, being independent of the number of flies used. The error bars

represent the standard error. It reports the uncertainty of the fractional survival and is calculated by the method of Greenwood. For the lifespans done with only 3 days at 29°C and then the vials transferred at 18°C, the flies were still counted three times per week but were transferred into a fresh vial only once a week.

2.2.2 Paraquat assay

4 to 5 days old adult flies raised at 18°C were transferred to 29°C for 3 days in standard food then put back to 18°C, into tubes containing filter paper soaked in 20mM paraquat diluted with sucrose, or just in water as a control. The flies were kept in a humid chamber and were counted every two to three hours during the day. Data analysis was done as previously described in Lifespan section.

2.2.3 Climbing assay

Flies were collected from the same cross used for the lifespan experiment in order to assess their negative geotaxis reaction. This assay is generally used to evaluate the motor capacity of the flies, even though the negative geotaxis reaction can be impaired by a loss of balance or geotaxis orientation loss. The flies were put into a graduated tube with four lines drawn every centimetre. The flies climbing above 4 cm were scored 4. The tubes containing each genotype were assessed at the same time using a home-made tool to bang down the tubes all together to avoid any differences in the strength of the banging movement. Each session were recorded to evaluate the scores. 5 females were assessed three times for each genotype. An average of the scores from the 5 females was made for each repeat. The average of the three repeats was then calculated. Statistical analysis was made by one way ANOVA using Dunnett's multiple comparison post hoc test. The experiment was repeated three times independently.

2.3 Tissue culture

2.3.1 S2 cell maintenance

Drosophila Schneider 2 (S2) cells were maintained in Schneider's medium supplemented with 1/100 dilution of antibiotics (5000 units/mL of penicillin and 5000 µg/mL of streptomycin solution, GIBCO) and 10% Fetal Calf Serum (FCS). The cells were cultured in either T25 or T75 flasks and split 1:10 when full confluence was reached. Cells were cultured in a 25°C incubator.

2.3.2 Transfections

Cells were split 1:20 from a full confluent T75 flask into a 6-well plate with 2mL of medium. Plasmids transfections were carried out using Effectene (Qiagen) following manufacturer's instructions. pMT-Gal4-GFP, pUAST-Luciferase-Luciferase 3'UTR, pUAST-Luciferase-Repo 3'UTR, pUAST-Luciferase-RepoΔ 3'UTR and pTK-Renilla were all used at 20ng/mL. pTub-miR-1 was used at 50ng/mL. The plasmid pBluescript was used at the same concentration as pTub-miR-1 to adjust equal total DNA transfected. The cells were cultured 2 days prior induction, which was done adding 500µM of copper sulphate to the medium.

2.3.3 Luciferase assay

The Luciferase assay was done 24h after induction. The cells were removed from the wells and transferred into Eppendorf tubes. 600µL were used for the Luciferase assay. The cells were spinned down, the medium removed and 75µL of PBS were added to each tube. The assay was then done using the Dual-Glo® Luciferase assay kit (Promega) according to manufacturer's instructions. The Luciferase and Renilla activities were measured in an opaque 96-well plate using a standard plate reader (Mithras LB 940, Berthod technologies). The Luciferase signal is normalized to the Renilla one. Then, the ratio between miR-1 and

no miR-1 conditions is calculated. Three independent transfections were averaged. Error bars represent standard error. Statistical significance was calculated with GraphPad Prism software using one way ANOVA and Dunnett's multiple comparison post hoc test. **: $p<0.01$

2.4 Dissections and Immunostaining

2.4.1 Imaginal discs staining

L3 larvae were anesthetized in cold PBS (see 2.7 Table of solutions). The head part of the larvae was dissected in cold PBS followed by the separation of the imaginal discs from the brain. They were transferred immediately into chilled PLP (see 2.7 Table of solutions) for 45 min at room temperature (RT). The discs were then washed in Wash Buffer (see 2.7 Table of solutions) for 15 min. They were blocked in wash buffer with 10% Fetal Bovine Serum (FBS) for an hour at RT in a 72-microwell plate. Primary antibodies were diluted in the blocking buffer and the discs were incubated overnight at 4°C in a humid chamber. The discs were washed three times 15 min in wash buffer and then incubated 2 hours at RT in secondary antibodies diluted 1/200 in blocking solution. After three washes of 15 min, they were washed in phosphate buffer without detergent and dehydrated overnight at 4°C in a PBS (20%)-Glycerol (80%) solution. They were finally mounted on slides in Vectashield®.

2.4.2 Adult brain and larval brain staining

Adult flies or L3 larvae were anesthetized on ice. Brains were dissected and put straight into 4% paraformaldehyde (PFA) for 45 min fixation. They were then washed in wash buffer for 30 min before blocking for an hour at RT in a 96-well plate. Primary and secondary antibody steps were similar to imaginal disc section with secondary antibodies being incubated overnight at 4°C. After three wash of 15 min, brains were put in Vectashield® in a 72-microwell plate overnight at 4°C. The brains were then mounted in

Vectashield® on a slide, surrounded by two coverslips on each side, before being covered by another coverslip on top. This mounting prevents the brains from being squashed by the top coverslip.

2.4.3 Embryos staining

Adult flies were laying eggs on apple juice plates with yeast paste. After aging the embryos to the desired stage at 25°C, they were collected and dechorionated with 50% bleach for 5 min and rinsed well with ddH_2O. The embryos were placed into a 1.5mL eppendorf tube containing heptane::PBS-formaldehyde (4%) in a 1:1 ratio (vol.:vol.). They were incubated 30 min rotating. The aqueous layer (PBS-FA) was removed and replaced by an equal volume of MeOH. The tube was vortexed for a minute for the embryos to pop out of their vitelline membranes and fall down to the bottom of the MeOH phase. The devitellinized embryos were then washed three times in MeOH to remove residual heptane. MeOH was replaced by PBS-T and rinsed three times in wash buffer. They were then blocked an hour at RT and then stained with primary antibodies overnight at 4°C followed by three wash and by secondary antibodies incubation overnight at 4°C as well. The embryos were washed three times in PBS-T and once in PBS before being mounted in Vectashield® the same way as for adult brains.

2.4.4 TUNEL assay

Fly brains were dissected in cold PBS and fixed for 45 min in PFA 4% at RT. After three wash of 10 min in PBS, the brains were incubated 10 min in 50 µL of Proteinase K (20µg/mL in PBS). The brains were washed twice 10 min in PBS to stop the permeabilization. For the positive control, a 10 min incubation in DNAse I buffer was followed by a 10 min incubation in DNAse I (7 U/mL in DNAse I buffer) and then washed three times in ddH_2O. The brains for all the conditions were then equilibrated 30 min in equilibration buffer and incubated 1h at 37°C with a mix solution (44 µL of equilibration buffer, 5 µL of marked nucleotide mix, 1 µL of TdT enzyme, Promega). The reaction was

stopped for 15 min in 2X SSC solution (diluted in ddH_2O) and washed three times in PBS. The brains were then mounted in Vectashield.

2.4.5 Dye injection

~0.5 µL of Fluorescein Dextran (50mg/mL, 10 kDa, Invitrogen, D1821) was microinjected into the soft tissue at the basis of the first pair of leg in anesthetized adult flies after 6 days spent at 29°C when the Repo-RNAi 1 flies start to have motor defects, or a specified time point for the Repo-RNAi 2 flies. After 18h, the heads are fixed 30 min in PFA 4%. Then, the proboscis were removed and the heads were fixed for another 30 min before brain dissection. The brains were then washed three times 20 min in PBS. Then, they were mounted straight after on a coverslip in Vectashield. Before taking the pictures, the settings were adjusted using a control sample in order to get reference parameters such as laser intensity, gain, offset and pinhole. All the pictures were then taken using these settings.

Confocal pictures were taken with an Olympus FV500, analysed with ImageJ Fiji software and processed with Adobe Photoshop 7.0.1.

Antibodies used: anti-GFP (1/500, rabbit polyclonal, Molecular Probes), anti-GFP (1/100, mouse monoclonal, Roche), anti-GFP (1/500, chicken, kindly provided by M.Meyer), anti-Repo (1/100, DSHB 8D12), anti-Srp (1/1000, rabbit polyclonal, kindly provided by A.Giangrande), anti-Elav (1/500, rat, DSHB 758A10 or 1/2000 mouse, DSHB, 9F8A9), anti-Atro (1/200, mouse monoclonal, Abmart), anti-ß-Gal (1/500, rabbit polyclonal, Molecular Probes, A11132).

2.4.6 Semi-thin retinal sections

Fly heads were cut after anaesthesia with CO_2. Female right eyes were kept for the experiments. Eyes were incubated in a fixative solution (2% glutaraldehyde in phosphate buffer 0,1M). Same volume of post-fixation solution was added (50% osmium, 50% phosphate buffer 0,2M). Samples were kept on ice for 30 minutes. The mix solution was

then replaced by a 100% post-fixation solution for 5 hours. A progressive dehydration by ethanol was proceeded prior to 2 propylene oxide washes of 10 minutes each at room temperature. Eyes were then incubated in a polymerisation solution (50% propylene oxide, 50% epoxy resin) overnight. Cut eyes were incubated in a 100% epoxy resin between 4 and 6 hours the following day. Eyes were then embedded in a mould filled with polymerization solution. They were then placed in the oven at 65-80°C overnight. After trimming, 1µm sections were performed by using a microtome (Leica Ultracut UTC). Sections were stained with 1% toluidine-blue in borax. Images were taken with light microscopy.

2.5 Molecular Biology

2.5.1 RNA extraction

50 heads were cut and placed into a 1.5mL eppendorf tube followed by a snap freeze in liquid nitrogen. The heads were then homogenized in 100µL of TriZol (Invitrogen) and 200µL of TriZol were topped up. The mix was incubated 10 min at RT before adding 60µL of Chloroform. The tubes were vigorously mixed during 20sec and then incubated 2 min at RT prior to 15min centrifugation at 4°C, 12000rpm. 200µL of the aqueous phase containing the RNA was taken out and transferred into another 1.5mL eppendorf tube and 150µL of isopropanol was added. A pellet of RNA will appear after 10 min centrifugation at 4°C and 12000rpm. The pellet was then washed three times with 75% EtOH and let dry before adding 40µL RNase free water with 1µL of RNasin® (Promega). The quality of the RNA extraction was assessed on a 1.5% agarose gel and the concentration and the 280/260 and 260/230 ratios were measured with a Nanodrop®.

2.5.2 DNA/RNA gels

DNA/RNA gel electrophoresis was done in TAE buffer (see 2.7 Table of solutions) using 1.5% agarose gel with 0.1µg/µL of Ethidium Bromide. DNA and RNA samples were

supplemented with DNA Loading buffer and run alongside 1kb or 100bp DNA ladder (New England Biolabs (NEB) or Promega) at 100V. DNA and RNA bands were visualized under UV light (Biospectrum® imaging system).

2.5.3 Cloning steps and DNA preparation

DNA digestions were done using restriction enzymes from NEB according to manufacturer's instructions. Linearized plasmid was dephosphorylated 3h at 37°C with phosphatase alkaline (New England Biolabs). DNA bands for future ligations were cut from the agarose gel on a UV-light platform and DNA was purified using QIAquick gel extraction kit (QIAGEN) according to manufacturer's instructions. The concentration of the DNA extracted was measured by NanoDrop®. Ligation was done using T4 DNA Ligase (NEB) according to manufacturer's instructions. The product of the ligation was transformed into competent bacteria (Invitrogen) according to standard transformation procedure. Bacteria were grown overnight at 37°C onto LB agar plate (see 2.7 Table of solutions) supplemented with antibiotics according to the resistant gene present in the plasmid. Several minipreps were made from single colonies cultured in 2mL LB (see 2.7 Table of solutions) supplemented with the appropriate selective antibiotics. The presence of the plasmid was assessed by a quick DNA extraction and purification using QIAGEN® plasmid mini kit reagents according to manufacturer's instructions. Appropriated restriction enzymes were used before running a DNA gel to evaluate the size of the bands from the plasmid extracted. A midiprep is then cultured from a good colony overnight at 37°C. The DNA is then extracted and purified using QIAGEN® plasmid midi kit protocol (QIAGEN) according to manufacturer's instructions. Its concentration and quality is assessed by NanoDrop®.

2.5.4 Genomic DNA extraction

Genomic DNA isolation was made out of one adult fly or one egg. They were placed into a 0.5mL eppendorf tube and homogenized with 50µL of proteinase K buffer (see 2.7 Table of

solution). The tube was incubated at 37°C for 30min. The proteinase K is then inactivated 95°C for 2min. 1µL is used for a PCR reaction. The tube is then stored at -20°C.

2.5.5 PCR reaction

PCR was done to identify the presence of endogenous or exogenous genes, using exons for primer design for endogenous genes. For the PCR of mfas, two sets of primers were designed. The first one, targeting the first exon, amplifies a product of 106bp and the second set, targeting the 4th exon, amplifies a product of 160bp. (See Table below). A master mix of PCR reagents was made when necessary. Otherwise, for each reaction, the following protocol was used: 5µL of Flexibuffer 5X (Promega), 0.5µL of dNTPS (10mM, Promega), 0.5µL of primers (10 pmol/µL, Eurofins Genomics), 1.5µL of $MgCl_2$ (25mM, Promega), 0.2µL of GoTaq polymerase (Promega) and 15.8µL of DNase/RNase free water. 1µL of the genomic DNA was added to the mixture. The PCR reaction program used was as following: 10min denaturation at 95°C, start a loop for 34 cycles, 30sec denaturation at 95°C, 30sec annealing at 56°C, 1min elongation at 72°C, end of the loop, 5min final elongation at 72°C and store at 8°C. 10µL of the PCR reaction is then loaded on a 1.5% agarose gel beside 6µL of 100bp DNA ladder.

Table 1: Primers used for PCR amplification of *mfas*

Name	Primer sequence
mfas 1st exon Forward	CCAACCGAGGCTCCAGATTA
mfas 1st exon Reverse	GCCGTAGAATGGAACCGC
mfas 4th exon Forward	CTGACTTCACAACGCCCTG
mfas 4th exon Reverse	GGCACGAAGAGGGTCACC

2.5.6 qPCR

1.5µg of each RNA sample was sent to the King's college London Genomic Centre Facility for processing. The qPCR was made using UPL probes and primers designed by the Universal Probe Library software (Roche) for quantification (see Table below). Three independent biological replicates were measured for each genotype of each time point. *Gapdh* and *eIF4A* were used as house keeping genes for controls. 9 genes were quantified, depending on the genotype and the time point.

Table 2: Oligos and UPLs used for qPCR

Gene	**CG number**	**UPL probe #**	**Left Oligo 5'-3'**	**Right Oligo 5'-3'**
repo	CG31240	65	GCATCAAGAAGAAGAAGACGAGA	GTTCAAAGGCACGCTCCA
loco	CG5248	116	CTGGTTTATCAACGCCTATGAA	GAGTGCGGAAGGAAGACTGT
Glio-tactin	CG3903	69	CGAATCGTCCAATTACAGAGC	GAAAAATTCCAGGAGAAACTGG
Gs2	CG1743	127	GCACCCTCGACTTCATTCC	GCACGAGCTTCCATCGTAGT
Pointed	CG17077	63	CTTTCTGTCCAGCCTAGTTGAGT	TGCACAGATCCTTGCATCC
dEAAT1	CG3747	30	GAATAAATTTGCTTGACATCCTTTT	AAAGCACGATTGGCAGTCA
wg	CG4889	81	GGCAAAATCGTTGATCGAG	GCAGGACTCTATCGTTCCTTCA
Gapdh	CG12055	18	AAAAAGCTCCGGGAAAAGG	AATTCCGATCTTCGACATGG
Gal4		139	CCCGACCATGATTACGGATA	TTGAAGTAAACGGGATGTTGTG
eIF4A	CG9075	104	CGTGAAGCAGGAGAACTGG	CATCTCCTGGGTCAGTTGGT
GFP		67	GAAGCGCGATCACATGGT	CCATGCCGAGAGTGATCC

2.6 Biochemistry

2.6.1 Fly head homogenisation

10 male and 10 female flies per genotype were decapitated with a scalpel, snap-freezed and homogenized using a pestel in 75µL of 1X Sample Buffer (SB) (see 2.7 Table of solutions) with 5% ß-mercaptoethanol freshly added. The tube was centrifuged at 12000 rpm at 4°C for 5min. 70µL of the supernatant was taken out and transferred into a new tube. The volume equivalent to 5 heads was loaded in each well.

2.6.2 SDS-PAGE

Protein samples were boiled 3min and spinned down prior loading. They were then loaded on 10% w/v polyacrylamide gels using BioRad gel electrophoresis apparatus. The samples were run at 60V while in the stacking part of the gel and then at 90V in the resolving part, constant voltage, in running buffer (see 2.7 Table of solutions)

2.6.3 Western Blot

After separation in the gel, proteins were transferred onto a nitrocellulose blotting membrane (Amersham™, Protran™, 0.2µm, GE Healthcare, Life sciences) for 1 hour at 60V, kept in cold, in transfer buffer (see 2.7 Table of solutions). The membrane was then blocked for an hour in 5% BSA or 5% milk in TBS-T (see 2.7 Table of solutions) at RT before incubation in primary antibody diluted in blocking buffer overnight at 4°C. The membrane was washed three times 15min in TBS-T at RT and then incubated 1 hour at RT in secondary antibody. After three wash in TBS-T, the membrane was washed again in TBS without Tween20. ECL reagent was mixed according to manufacturer's instructions (SuperSignal WestPico or ECL Western blotting substrate, Pierce) and put onto the membrane to fully cover it. After 3min of incubation, the ECL liquid was removed and the membrane placed between two transparent plastic sheets and transferred into a cassette.

Once into the dark room, a film was disposed into the cassette for a certain amount of time, depending on the strength of the signal, and then transferred to a developer. The film was then scanned and processed using Adobe Photoshop 7.0.1 and the bands were quantified using Image Studio Lite 4.0.

Antibodies used:

Anti-Repo (mouse monoclonal, 1/200 in blocking solution, DSHB 8D12), anti-GAT (rabbit polyclonal, 1/10000 in 5% milk-TBS-T, gift from Marc Freeman), anti-ß-actin (rabbit polyclonal, 1/3000 in blocking solution, SAB 21338), anti-myc (mouse monoclonal, 1/1000 in blocking solution, Roche 9E10), anti-GFP (mouse monoclonal, 1/1000 in blocking solution, Roche).

2.7 Appendix

Table 3: Solutions used in this study

Solutions	Compositions
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO_4, pH 7.2
TBS	50 mM Tris-HCl, 150 mM NaCl pH 7.6
TAE	40 mM Tris-HCl, 20 mM acetic acid, and 1mM EDTA
TBS-T	TBS with 0.1% v/v Tween20 (Calbiochem)
Wash buffer	PBS + 0.2% Tx-100
PLP	1/4 8% paraformaldehyde and ¾ lysine (0.12 mol/L) with Na_2HPO_4
Blocking solution	TBS-T with 5% BSA
Running Buffer	25 mM Tris, 0.193M Glycine and 0.1% SDS
Transfer buffer	25 mM Tris, 0.193M Glycine, 10% v/v MeOH
4X Sample buffer	200 mM Tris-HCl (pH 6.8), 6.4% v/v SDS, 16% v/v glycerol, 0.25% w/v bromophenol blue
Proteinase K buffer	10 mM trisCl pH 8.2, 1mM EDTA, 25 mM NaCl and 200 µg/mL of Proteinase K (Invitrogen) freshly added
LB medium	1 % w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl (pH 7)
LB agar	LB medium containing 1.5% w/v agar

Chapter III:

Effect of polyQ Atro expression in glial cells

3.1 Introduction

Drosophila melanogaster has been proven to be an excellent model to study neurodegenerative diseases. A lot of different models have been established to study specific genetic diseases such as ALS/FTD, Parkinson's disease, Alzheimer's disease or Huntington's disease (Muqit et al. 2002, Hirth 2010, McGurk et al. 2015, Zhang et al. 2015). The powerful genetic tools available for *Drosophila* offer the possibility to express mutated genes or any other transgene in specific subtypes of cells in a tightly controlled manner. The use of different systems such as UAS/Gal4, LexAop/LexA combined with a temperature sensitive Gal80 (Gal80ts) allows the expression of different genes in different cell types and thus deciphering cell-cell interactions. Moreover, *Drosophila* models have also been established to study recessive diseases. It is the case for Parkinson's disease for instance, with the study of *parkin* and *pink* mutants. Using loss of function (LOF) or knockdown by RNAi of recessive genes could provide useful models as it is the case for recessive familial Parkinson's disease (Muñoz-Soriano et al. 2011).

However, most of the research in the field so far has focused on the neuronal contribution to the phenotypes they observe for a particular disease. Indeed, a common pattern between all neurodegenerative diseases is the actual loss of neurons in specific part of the brain. This specificity will give rise to distinct symptoms coming from the function of the brain region affected. Another pattern commonly observed in many neurodegenerative diseases is exacerbated gliosis in specific regions of the brain (Pekny et al. 2005, Sargin et al. 2009, Schwab et al. 2010). However, whether it is protective or harmful for the neurons in those regions is still open to debate (Jha et al. 2012). DRPLA is not an exception in that sense, as mentioned earlier in Chapter I.

A recent DRPLA fly model established in our group has been used to understand the interaction between *polyQ Atro* (and human *polyQ Atrophin-1*) and the autophagy pathway. Beside the discovery of a link between *polyQ Atro* expression and the blockage of the autophagic flux, this study has revealed for the first time a potential contribution of glial cells to the pathology. The expression of *polyQ Atro* has been shown to be toxic in neurons

and glial cells, even when the expression is limited to the adult stage of *Drosophila*. Indeed, *polyQ Atro* expression from developmental stage compared to adult specific expression showed a similar phenotype (Nisoli et al. 2010). The drivers used for this study were *Elav-Gal4*, a pan-neuronal driver and *dEAAT1-Gal4*, a driver commonly used to target astrocyte-like glia using the promoter of the only glial glutamate transporter present in *Drosophila*, known to be expressed in the neuropil (Seal et al. 1998, Besson et al. 1999, Kawano et al. 1999). This study also shows that expressing the human mutated *Atrophin-1* in glia or in neurons has a similar phenotype to what is observed with *Drosophila polyQ Atro*. However, using the same drivers for glial or neuronal expression, the expression of *polyQ huntingtin*, the mutated protein responsible for Huntington's disease, showed an inversion of the drivers' strength. The neuronal one has a stronger phenotype than the glial one, contrary to the opposite effect observed with *polyQ Atro*. Taking together, these results show that in comparison to another polyQ protein, the balance of toxicity is more towards the glial cells for *polyQ Atro*.

Not all the brain regions are affected in the same way for each neurodegenerative disease. It remains unclear why some neurons are more sensitive than others to the expression of mutated proteins. Depending on their functions, their gene expression profile or their connections to other regions of the brain, neurons can be affected and then die due to this particular mutation, or just survive and function normally. It is now well established that glial cells contribute to neuronal functions, regulating their excitation rate by acting at the synaptic level, or providing the neurons with energy, using the well characterized lactate shuttle for instance (Bélanger et al. 2011, Pannasch et al. 2014, Tang et al. 2014). It is then easy to understand that if glial cells are also affected by these mutations, it can trigger some dysfunctions that can affect directly or indirectly neurons (Volkenhoff et al. 2015). In the case of DRPLA, as mentioned above, the mutated glial cells seem to be sensitive to the toxicity of the autonomous glia specific *polyQ Atro* expression. However, the questions are: are all the glial cells equal to the sensitivity to these mutated proteins? Or, are some, similarly to neurons, more affected than others, and thus having a more important role on the disease progression?

The aim of this chapter is to understand whether all or some glial subpopulations are affected by the expression of *polyQ Atro*. And from the ones that are affected, which ones seem to be more important. Using different glial cell drivers, specific to the different subtypes of glia present in the *Drosophila* adult brain, *polyQ Atro* was expressed specifically in subsets of glial cells. Lifespan was used as readout in order to assess the general impact of the mutated glia on the organism function.

3.2 Results

3.2.1 Glial subtypes display different sensitivity to *polyQ Atro* toxicity

In order to characterize *polyQ Atro* toxicity on glial subtypes, I first wanted to see its effect when the mutated protein was expressed in all adult glial cells. Lifespan assay was used to monitor the toxic effect on the glial cells and the impact on the organism. To this end, the pan-glia driver *Repo-Gal4* was used, which uses the promoter of the *repo* gene, expressed in all glial cells from development to adulthood, apart from midline glia, which are present only during embryogenesis. For these sets of experiments, $Gal80^{ts}$ ("temperature sensitive", inhibiting Gal4 at 18°C but unfolded at 29°C) was not used as it has been shown that the expression of *polyQ Atro* in glial cells during development did not influence the adult lifespan of the *Drosophila* using either *dEAAT1-Gal4* (Nisoli et al. 2010) or *Repo-Gal4* at least (in this study). The crosses between the driver and the *UAS-Atro75QN* (polyQ Atro line) or w^{1118} (control) were kept at 18°C in order to keep the *Gal4* activity as low as possible during development even though the adult does not seem to be affected. Once the progeny hatched from the pupae, they were collected and transferred to 29°C (see **Chapter II** for Materials and Methods).

The lifespans were analysed using the software Prism GraphPad and the Kaplan-Meyer method (using the log-rank test). In addition to the P-value, this method also provides a Chi square value (χi^2), used to calculate the P-value, both being dependent from the number of flies used in the assay. Thus these values can be directly compared only when the same number of flies is used. While the P-value is used to determine the statistical significance of an experiment, the χi^2 value will be used in this thesis to rank the strength of the phenotype, being directly proportional to the difference between the curves. Indeed, it is more intuitive than the P-value, inversely proportional to the difference between the curves. However, this value provides only an indication of the strength and will be used in addition to the median survival of each curves, which is independent of the number of flies used. From the experience acquired in the lab on lifespan experiments, the use of two sets of 60 flies

genetically identical can give a P-value statistically significant. It demonstrates the variability of the assay and the limit of the use of the P-value to assess the biological significance. Thus, for the rest of the thesis, I will use the χi^2 value and the median survival to compare different phenotypes but it will only provide an indication for the strength of a phenotype but not the statistical or biological significance of it.

The lifespan of the flies expressing *polyQ Atro* in all the glial cells is dramatically reduced compared to the control lifespan (**Figure 3.1A**). Indeed, the median survival of the *polyQ Atro* expressing flies is 14 days compared to 34 days for the control. The corresponding χi^2 value is 111. This reduction by 60% of the lifespan highlights the importance of glial cells to maintain a functional organism. However, in this set up, polyQ Atro protein is expressed from development. Thus, it is also possible that some developmental defects can take part of this phenotype. Furthermore, the comparison of the median survival obtained using *Repo-Gal4* to the one published by Nisoli et al. using *dEAAT1-Gal4* in the same conditions shows a similar strength of phenotypes. Its median survival is 16 days compared to the control one of 30 days. This difference of only 2 days between *dEAAT1-Gal4* and *Repo-Gal4* drivers shows the high importance of the *dEAAT1* positive cells in the toxic effect due to *polyQ Atro* expression. However, *Repo-Gal4* driver being pan-glial, it will be used as a reference phenotype for further glial subtypes comparison.

Another line of *dEAAT-Gal4* was then assessed and compared to the one used in the past. The evaluation of a similar driver but inserted in another locus in the genome allows a more accurate evaluation of the driver's effect. Indeed, depending on its insertion, different levels of *Gal4* expression can be obtained using the same promoter. This variation can result in different strength of phenotype. In the case of *dEAAT1-Gal4*, two different lines located on two different chromosomes, one on the second and one on the third chromosome, were assessed. Using the same *UAS-Atro75QN* and w^{1118} lines, the strength of both insertions can be directly evaluated. The *dEAAT1-Gal4* inserted on the third chromosome has a median survival of 15 days when crossed to *UAS-Atro75QN* compared to 36 days when crossed to w^{1118} as control (**Figure 3.2B**). However, the one inserted on the second chromosome has a median survival of 20 days for *UAS-Atro75QN* with a control median survival of 30 days.

The Chi square obtained for those two lines are respectively 109 and 42 for *dEAAT1-Gal4* on the II and on the III chromosome. More than the tail of the curve of *dEAAT1-Gal4* III, the difference is also due to the fact the lifespans of the control and the *UAS-Atro75QN* are crossing each other from 6 to 16 days, decreasing the Chi square value, which is proportional to the area between two curves. In conclusion, when comparing the strength of the phenotypes using the Chi square values, the *dEAAT1* driver on the II chromosome is stronger than the one on the III.

Another way to characterize a cell population is to use different drivers meant to target the same population. A driver known to be specific to the astrocyte-like glia also targeted by *dEAAT1-Gal4* is *Alrm-Gal4*. *Alrm* codes for Astrocytic leucine-rich repeat molecule. Its function is not known, however its expression profile matches exactly the astrocyte-like glial cell population (Doherty et al. 2009). Similarly to *dEAAT1-Gal4* driver, two different lines of *Alrm-Gal4* are available, one on the second chromosome and one on the third. As seen in **Figure 3.1C**, both lines have the same phenotype when expressing *UAS-Atro75QN*, with a median survival of 26-27 days. However, when crossed to w^{1118} as control, they are slightly different. The insertion on the II chromosome has a median survival of 36 days while the one on the III has a median survival of 34 days. The respective Chi square values of *Alrm-Gal4* II and *Alrm-Gal4* III are respectively 49 and 33 between *polyQ-Atro* and w^{1118} curves, which is half of what was obtained using *dEAAT1-Gal4* II driver, which is supposedly targeting the same glial cell population. In the attempt to avoid any problems due to the strength of each promoter, which could explain this difference, a *Drosophila* line having both *Alrm-Gal4* was generated. This line was crossed to a recombined double *UAS-Atro75QN* line to avoid any limiting effect from the *UAS*. However, the lifespan of this double *Alrm-Gal4* does not increase the phenotype compared to one *Alrm-Gal4* only even though the Chi square values are different. The *polyQ Atro* lifespan is compared to either the drivers crossed to w^{1118} or the double UAS crossed to w^{1118}. The respective Chi square values are 80 and 53 (**Figure 3.1C**). It shows the variability of the Chi square value and the need to evaluate the median survival as well. The Chi square value is good for screening phenotypes but the median survival provides a better accuracy of the phenotype strength. This difference could be the result from a potential leakage of the *UAS-Atro75QN*

transgenes. The fact that the *dEAAT1* drivers and the *Alrm* ones have different strength could reflect different cell populations targeted by *dEAAT1-Gal4* drivers, which are not targeted by *Alrm-Gal4* ones, inside or even outside the CNS.

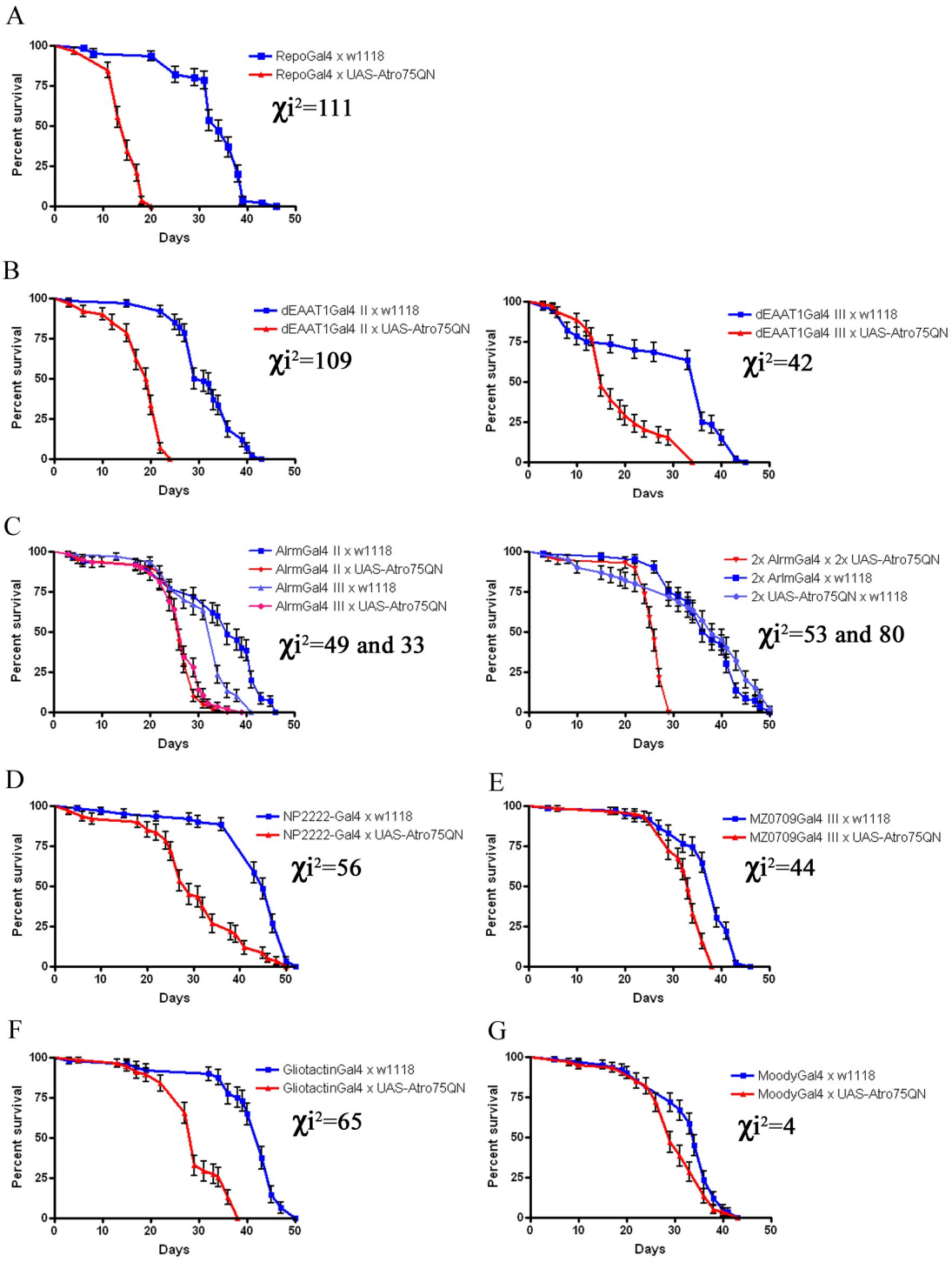


Figure 3.1: Effect of glial *polyQ-Atro* expression on *Drosophila* lifespan. (A) Lifespan of the pan-glial driver *Repo-Gal4* expressing *polyQ Atro*. **(B)** Comparison between *dEAAT1-Gal4* on the II chromosome (left panel) and *dEAAT1-Gal4* on the III chromosome (right panel) expressing *polyQ Atro*. **(C)** Comparison between both *Alrm* drivers on the II or III chromosome (left panel) and both together (right panel) expressing *polyQ-Atro*. **(D-G)** Lifespan of the other drivers used for this study **(D)** *NP2222-Gal4*, **(E)** *Gliotactin-Gal4*, **(F)** *MZ0709-Gal4* and **(G)** *Moody-Gl4*, all expressing *polyQ-Atro*. Lifespans are done at 29°C with 0-5 days old adult flies. 60 flies are used for each lifespan. Error bars represent standard error. w^{1118} is used as a control line. Statistical analysis was done with the software GraphPad Prism using the logrank test method.

To verify this hypothesis, a staining of the brains from the Gal4 drivers crossed with the reporter *UAS-CD8-GFP* was done. The GFP signal from *dEAAT1-Gal4* shows GFP positive cells that can be seen located all around the brain, and not only surrounding the neuropil (**Figure 3.2A**). *Alrm-Gal4* driver expressing the GFP reporter clearly shows cells surrounding the neuropil and sending projections in its direction (**Figure 3.2B**). These pictures confirmed the previous observations done by the MARCM (Mosaic Analysis with a Repressible Cell Marker) technique using *Alrm-Gal4* driver to identify and characterize the astrocyte-like population (Doherty et al. 2009). However, cells with star shape, having processes like *Alrm-Gal4* positive cells, are observed but these are not the only ones present when the *dEAAT1-Gal4* driver is used. In some specific areas, GFP signal surrounding potentially neuronal cell bodies or axonal bundles in the optic lobes is also present, suggesting other populations of glial cells expressing *dEAAT1-Gal4*. These populations can be identified with the ensheathing glia and cortex glia. This difference in the cell populations targeted could explain, at least partially, the different phenotype strength between the drivers.

These other potential glial subtypes, surrounding neuronal cell bodies and/or axonal bundles, look like the so-called cortex glia and neuropil ensheathing glia, characterized by MARCM technique using the drivers *NP2222-Gal4* (Gal4 insertion into the *Akap200* gene) and *MZ0709-Gal4* respectively (Doherty et al. 2009, Awasaki et al. 2011). I confirmed the specificity of these drivers using again the *UAS-CD8-GFP* reporter. The so called "cortex" glia is present only in the "cortex" part of the brain, surrounding neuronal cell bodies and their membranes are absent from the neuropil, providing an opposite picture to the *Alrm-Gal4* positive glia (**Figure 3.2C**). The neuropil ensheathing glia send projections to surround the glomeruli within the neuropils, being the limit between the astrocyte-like glia and cortex glia membrane domains (**Figure 3.2D**). Furthermore, at the level of the optic lobe, the ensheathing glia membranes form a specific pattern of axonal bundles reminding the shape of the cortex glia surrounding the cell bodies. However, by costaining with DAPI, this pattern does not contain nucleus and might represent aligned tubular structures formed by axonal tracts specific of the optic lobe neuropils (Pimentel C. and Fanto M., unpublished).

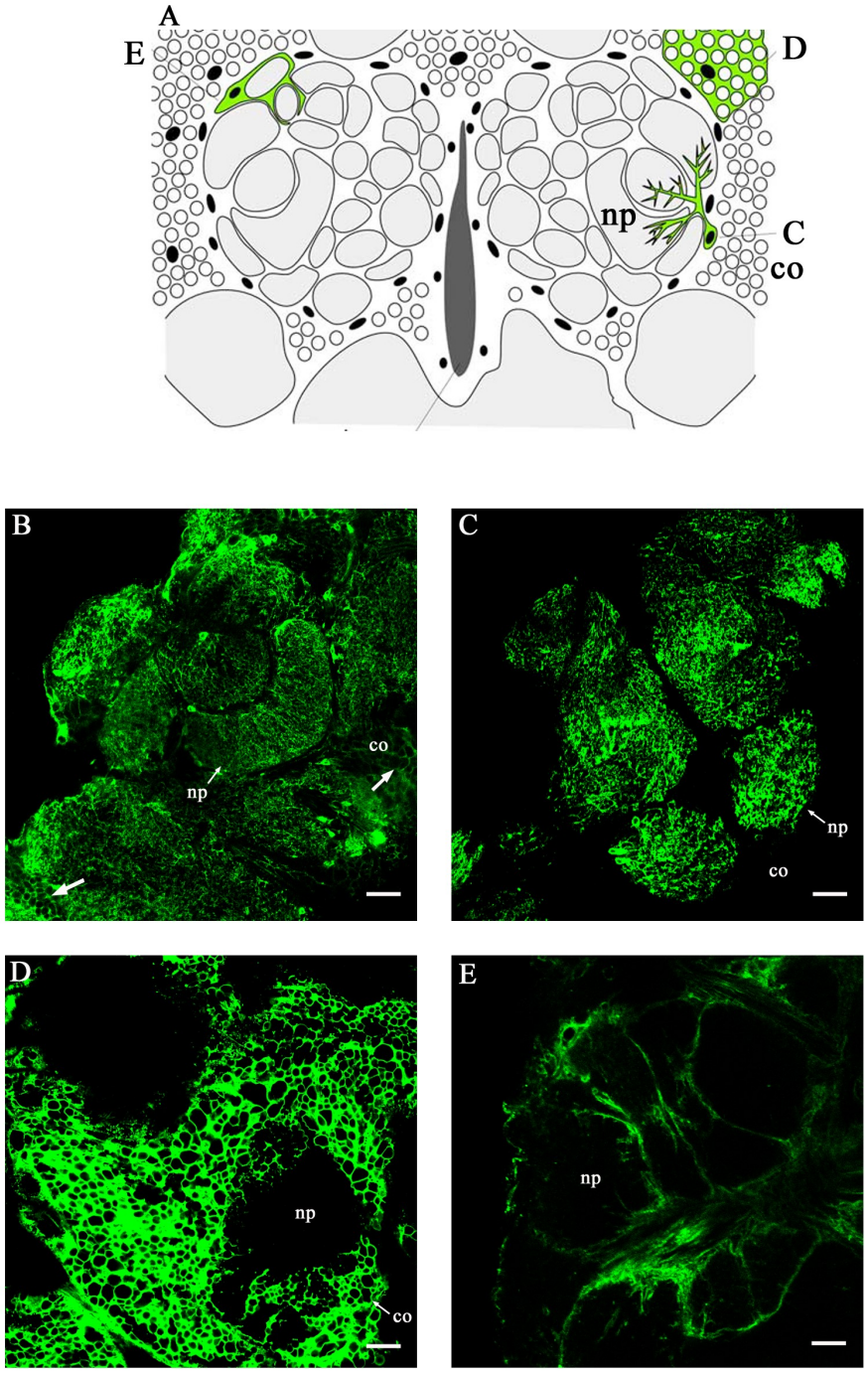


Figure 3.2: Expression pattern of different glial subpopulations using mCD8::GFP. Confocal images of different drivers crossed with *UAS-mCD8::GFP*, live signal from GFP. co: cortex, np: neuropil. **(A)** Schematic representing the different glial cell types and their morphologies, observed in the following images, adapted from Doherty et al. 2009, **(B)** *dEAAT1-Gal4*, **(C)** *Alrm-Gal4*, **(D)** *NP2222-Gal4*, **(E)** *MZ0709-Gal4*. Arrows indicate membrane GFP surrounding neuronal cell bodies. Scale bar: 10µm.

Using MARCM to label only one cell, it has been demonstrated that one "cortex" glia could ensheath a high number of neuronal cell bodies. Thus, *NP2222-Gal4* driver has then been used to characterize the effect of *polyQ Atro* on this glial subtype, which shows similar cell shape to some observed in the *dEAAT1-Gal4* staining. When crossed to the *UAS-Atro75QN*, the median survival is 29 days, compared to 45 days for the control (**Figure 3.1D**). Using the Kaplan-Meyer method, it gives a Chi square of 56. The phenotype of *NP2222-Gal4* driving *polyQ Atro* is also weaker than the *dEAAT1-Gal4* one. However, it is close to that of *Alrm-Gal4*, even though the control for *NP2222-Gal4* lives much longer. A possible difference in genetic background could be responsible for this effect. The targeted cell populations are completely separated, having different functions in the CNS. However, they seem to have an equal sensitivity to the expression of *polyQ Atro* and a similar effect on the *Drosophila* lifespan. However, only one Gal4 line is available for the *NP2222-Gal4* driver, limiting the interpretation of the results. In order to complete the study of the glial cells being present around the neuropils, I also evaluated the sensitivity of the ensheathing glia to the toxicity of *polyQ Atro*. Unlike astrocyte-like glia, ensheathing glia surround the neuropil glomeruli but do not closely associate with synapses unlike astrocyte-like glia (**Figure 3.2D**). Those cells remain poorly studied in the adult *Drosophila* CNS, however it has been demonstrated that they function as phagocytes in larvae to clear degenerating axons in injured brain through the *Draper*/*dCed-6* signalling pathway (Doherty et al. 2009). One driver has been characterized to be specific to ensheathing glial cells, called *MZ0709-Gal4*. When crossed to *UAS-Atro75QN* line or w^{1118}, its median survival is 33 days for the *Atro polyQ* and 39 days for the control (**Figure 3.1E**). This difference between the lifespan gives a Chi square value of 44. This value is in the range of *Alrm-Gal4* even though the phenotype seems weaker comparing their respective median survival. Moreover, similarly to *NP2222-Gal4*, only one driver line has been assessed for this cell population, which limits the interpretation of the results. Thus, its weak phenotype could be due to a weak Gal4 expression caused by a positional effect of the transgene or a weak expression level from the promoter itself.

In addition to the shortening of the lifespan of glial *polyQ Atro* expression, climbing defects have also been previously reported (Nisoli et al. 2010). This alteration of the motility could come from different CNS dysfunctions but also developmental defects or impairment of sensory organs. Astrocyte-like glia regulate the synaptic activity, thus impairment at this level could trigger this phenotype. However, it has also been reported that a defective insulation of the nervous system results in a similar behaviour (Kim et al. 2010). The *Drosophila* BBB is composed of two layers. The outer one, in direct contact with the hemolymph, is made by perineurial glia while the inner one, directly protecting the neurons and the cortex glia, is composed of subperineurial glia. The first layer does not form tight junctions and has a role of pre-filtering big molecules. However, the subperineurial glia forms tight septate junctions that prevent non-required molecules to get inside the brain (Schirmeier et al. 2015). *Gliotactin-Gal4* was used first to drive the expression of *UAS-Atro75QN* in the subperineurial glia of both CNS and PNS. *Gliotactin* codes for a transmembrane protein required for the septate junction development and especially for the formation of the blood-nerve barrier (Auld et al. 1995). The Gal4 driver using its promoter is used to target peripheral glia. However, a recent study using *UAS-CD8-GFP* as a reporter has shown that it is also expressed in the subperineurial glia of the CNS (Zwarts et al. 2014). When crossed with *UAS-Atro75QN*, *Gliotactin-Gal4* lifespan has a median survival of 29 days. Its control lifespan has a median survival of 43 days (**Figure 3.1F**). When it is around 33 to 37 days for the previous drivers used, *Gliotactin-Gal4* and *NP2222-Gal4* have a control median survival of respectively 43 and 45 days. The Chi square of this subperineurial glia driver is 65, compared to 56 for *NP2222-Gal4*. Another "BBB" driver specific to subperineurial glia called *Moody-Gal4* was also assessed. *moody* encodes for two G-protein coupled receptor proteins (GPCRs) that are required continuously to maintain the BBB integrity (Bainton et al. 2005). As previously done with the other drivers, the driver was crossed to either *UAS-Atro75QN* or w^{1118} flies. The difference between the two lifespans is milder than the other drivers assessed so far (**Figure 3.1G**). The median survival of the *UAS-Atro75QN* is 29 days compared to its control of 34 days. The Chi square value obtained for *Moody-Gal4* is only 4, the weakest value of all drivers used for this study. However, as mentioned before, its p-value is statistically significant (p=0.023). It is possible that the level of Gal4 expression by *moody* promoter is a lot weaker than

Gliotactin promoter. Thus, it is possible that the sensitivity of subperineurial glia to *polyQ Atro* expression participates to the observations made with *Repo-Gal4* on motor defects and overall lethality.

According to the drivers used and the ranking of their Chi square values, the ensheathing glia, via the *MZ0709-Gal4* driver, seem to be the less convincing cell type to follow up as being important in the reduction of the lifespan observed by a pan-glial expression of *polyQ Atro* (summary **Table 4**). Indeed, there is only 6 days difference in the median survival between the polyQ and control flies. However, only one line was assessed for this subtype, which limits the confidence in the phenotype observed. Also, for subperineurial glia, both drivers assessed have very different phenotypes, which could lead to different interpretations on the role of this glial subtype in the *polyQ Atro* toxicity. So far, the most convincing phenotypes come from Gal4 drivers being expressed in astrocyte-like glia, *dEAAT1* driver on the II chromosome being the strongest after the pan-glia *Repo-Gal4*. To this end, I decided to further analyse the population targeted by *dEAAT1* drivers by generating new *dEAAT1* tools to allow this study.

Drivers	Glia subtypes	Chi square
Repo-Gal4	Pan-glial	111
dEAAT1-Gal4	Astrocyte-like glia and Cortex glia/Ensheathing glia	42 to 109
Alrm-Gal4	Astrocyte-like glia	33 to 80
NP2222-Gal4	Cortex glia	56
Gliotactin-Gal4	Peripheral glia	65
MZ0709-Gal4	Neuropil ensheathing glia	44
Moody-Gal4	BBB glia	4

Table 4: Summary of phenotype strength for each glia subtypes analysed. The Chi square value is given by GraphPad Prism software using the Kaplan-Meier method and log-rank test analysis.

3.2.2 *dEAAT1* positive cells are not only astrocyte-like glia

Expressing *polyQ Atro* in *dEAAT1* positive cells has the strongest phenotype after *Repo-Gal4* expression. *dEAAT1* is a glutamate transporter and is thought to be astrocyte-like specific in the CNS. However, the mCD8-GFP staining shows a different pattern between *Alrm-Gal4* and *dEAAT1-Gal4*. *dEAAT1-Gal4* phenotype is relatively similar to *Repo-Gal4* having a Chi square of about 100 each. However, while *Repo-Gal4* targets all adult glial cells, *dEAAT1-Gal4* is supposed to be expressed only in glia invading the neuropil and a subset of neurons present in the lamina of the optic lobe (Rival et al. 2004). It raises the following question: what are the subsets of cells expressing *dEAAT1-Gal4* that are responsible for the toxic effect? The characterization of this population could help us understand the role of each glial subtype in the polyQ toxic effect on the organism. The use of this driver instead of *Repo-Gal4* could allow us to address more specific questions on physiological problems that occur due to *polyQ Atro* expression, which ultimately result in the fly death.

3.2.2.1 Generation of new *dEAAT1* driver lines

To address this question, I decided to generate two different drivers using the *dEAAT1* promoter from the *dEAAT1-Gal4* plasmid. The first one is *dEAAT1-LexA-GAD* (Gal4-Activating-Domain). LexA-GAD binds to a sequence called *LOT* (also called *LexAop*), in the same way UAS/Gal4 system does. The LexA DNA binding domain is specific to its upstream activating sequence (from genes known to be activated by LexA), which in this case comes from *E.coli* instead of yeast for UAS/Gal4. The LexA sequence used in this study comes from an improved form of its former toxic sequence, which also had weak expression levels. The addition of the Gal4-hinge domain (hinge domain referred later as H, a short stretch enriched in acidic amino acids, from aa 148 to 196 of Gal4, known to serve as auxiliary activation domain in yeast) and the modifications of the activating domains (shorter GAD or VP16 in place of the formerly developed version) have increased the efficiency and decreased leakage and toxic effect. The *GAD* sequence used to add downstream *LexA* is a short version of the Gal4 GAD (amino acid 768 to 881 of Gal4),

which is the minimal sequence required to activate the transcription (Yagi et al. 2010). The use of GAD as transactivator allows the inhibition of the LexA activity by the use of Gal80. It provides a tight control of its activation in time, using $Gal80^{ts}$, but also a more specific expression in subset of cells, by potentially using Gal80 lines that would partially overlap with *dEAAT1-Gal4* expression. The second one is *dEAAT1-LexA-VP16*. The VP16 protein comes from the herpes virus and its activating domain has been restrained to a small sequence in order to be incorporated downstream the DNA-binding domain (minimal activation domains from VP16 from positions 436 to 447, put twice in a row according to (Baron et al. 1997)). The construction of such driver allows a transgene expression independently from Gal80 activity.

The constructions of the plasmids were made using *dEAAT1-Gal4* sequence incorporated into a *pCASPER4* plasmid (see map **Figure 3.3A**), *LHV2* insert (LexA:H:$(VP16mAD)_2$) and *LHG* insert (LexA:H:GAD). First, I wanted to confirm the location of XbaI restriction sites that would be later used to remove *Gal4*. XbaI and SpeI restriction sites were cut to confirm their presence and their good locations in the plasmid (**Figure 3.3A**). The XbaI restriction enzyme digestion of the plasmid gives rise to 3 bands. The top one is the linearized plasmid *pCASPER4* with a size of around 14kb. The middle band is the 5kb 3' end of the *dEAAT1* promoter (which is 8.5kb overall) and the lower band just above the 3kb ladder correspond to the *Gal4* sequence with the *hsp70* 3'UTR region. The digestion using SpeI gives unexpectedly 3 bands instead of two. The top one is the *pCASPER* plasmid linearized and the middle one at a size of 3.5kb should be the *Gal4* sequence. However, a band of 2kb is also present. The plasmid was then digested using both restriction enzymes at the same time to be able to confirm the unknown Spe site that seems to be within the *dEAAT1* sequence. As expected, the product of the digestion was 4 bands. The 5kb band from the *dEAAT1* sequence is cut into 2.8kb and 2kb sequences, surrounded by XbaI sites. A band at 200bp is actually also expected but is visible only with a high UV exposure (**Figure 3.3A**).

To remove the *Gal4* sequence, the restriction enzyme XbaI was used. However, as previously mentioned, this enzyme will cut the 5kb of the *dEAAT1* promoter. Thus the cut

was performed in two different tubes, one being dephosphorylated to avoid the vector to re-ligate itself (**Figure 3.3B**). The middle band phosphorylated and the top band dephosphorylated, corresponding respectively to the 5kb lacking of *dEAAT1* and *pCASPER4* were extracted from the gel and ligated to form *pCASPER4-dEAAT1* plasmid. The selected transformed bacteria were then checked using SpeI and NotI restriction enzymes in order to confirm that the 5kb *dEAAT1* fragment has been correctly inserted back (**Figure 3.3C**).

The following step was to insert the *LHV2* sequence into the *dEAAT1* vector using NotI restriction sites. *dEAAT1* vector has been dephosphorylated, and both *dEAAT1* and *LHV2* plasmids were cut using NotI. The *dEAAT1* vector and *LHV2* fragment were then extracted from the gel and ligated. After bacteria amplification, the ligation was controlled using either KpnI or NotI for the presence of the *LHV2* insert in the good direction (**Figure 3.3D**). The plasmid was then amplified and purified to be sent to inject. Its concentration after the purification step was 944 ng/µL.

The replacement of *LHV2* by *LHG* was done using NotI restriction sites. *LHV2* was first excised. *LHG* and *dEAAT1* were cut with NotI and in a similar manner than previously, were ligated, amplified from the good bacterial colonies (**Figure 3.3D**) and purified. The final concentration of the *dEAAT1-LHG* plasmid ready for the injection was 1345 ng/µL.

Both plasmids were sent to inject into fly embryos. The larvae that have survived the injection and became adult were crossed to w^{1118} flies. Using standard procedures with eye colour phenotype for the insertion of the plasmid (the plasmid contains the mini-white "*w*" gene) and double balancers to identify the chromosome in which it is inserted, I isolated 5 different lines of *dEAAT1-LexA::GAD* and 4 *dEAAT1-LexA::VP16*. Out of the 5 *LexA::GAD*, one is on the II chromosome, 3 on the III and one on the X. Two on the III are homozygous lethal. For the *LexA::VP16*, two lines are on the second and two on the third. One line on the second chromosome is homozygous lethal. The others are viable.

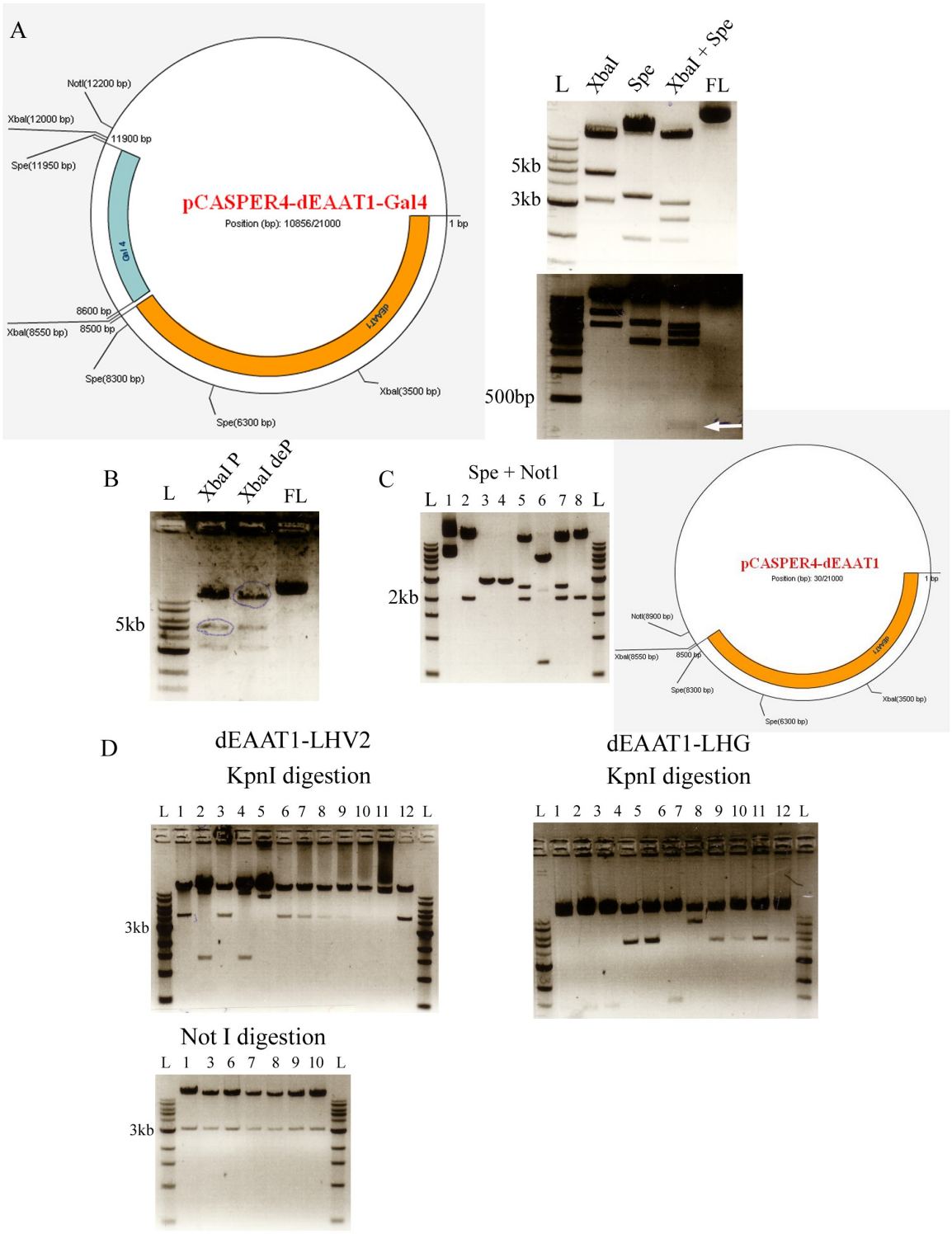


Figure 10: Generation of *dEAAT1-LexA* plasmids. (A) Left panel: annotated map of the *dEAAT1-Gal4* plasmid used as backbone. Right panel: Digestion of the plasmid by XbaI and Spe restriction enzymes to confirm the sites. Arrow on the lower gel indicates the 200bp band visible with high exposure. **(B)** Digestion of the *dEAAT1-Gal4* plasmid by XbaI and partial dephosphorylation (second well) to religate the cut portion of *dEAAT1* in the process. **(C)** Digestion of miniprep colonies from *dEAAT1* plasmid and updated annotated map. **(D)** Left panels: Digestion of the plasmid *dEAAT1-LHV2* from minipreps by KpnI and NotI to confirm the presence of the *LHV2* construct. Right panel: Digestion of *dEAAT1-LHG* with KnpI to confirm the correct insertion of *LHG*.

3.2.2.2 *dEAAT1* positive cells are not only different types of glial cells but also neurons

First, I verified that the *dEAAT1-LexA* was indeed activating transgenes downstream *LOT* sequence in a similar pattern than *dEAAT1-Gal4*. To this end, *UAS-nls-GFP* and *LOT-mCherry* were used as reporters and respectively combined to *dEAAT1-Gal4* and *dEAAT1-LexA::GAD1* drivers. An identical pattern was confirmed by colocalization of GFP and mCherry signals in adult brain (**Figure 3.4A**). The lifespan phenotype of all the LexA lines was then assessed. They were crossed with either *LOT-polyQ-Atro* or *LOT-wt-Atro*. The construct *LOT-wt-Atro* was used as a control because it was built beside the *LOT-polyQ-Atro* and is a better control for the toxicity of polyQ. All the phenotypes observed using *LexA::GAD* were weaker than the ones using *dEAAT1-Gal4*. This is not due to the difference between *UAS-polyQ-Atro* and *LOT-polyQ-Atro* since using respectively *Repo-Gal4* or *Repo-LexA*, it gives the same strength of phenotype (compare **Figure 3.1A** and **4.1C** in Chapter IV). The lines with the strongest phenotypes were *dEAAT1-LexA::GAD* 1 and 3 which have respectively a Chi square of 70 and 35, and a median survival of 27 days and 22 days (**Figure 3.5A**). The Chi square of the *GAD* 1 line is double the one of *GAD* 3 because of the extended lifespan of its control with wt Atro, which has a median survival of 38 days, compared to 30 days for the *GAD* 3 line. The lifespan of the *dEAAT1-LexA::VP16* could not be assessed since no adult progeny were obtained when crossed to *LOT-polyQ-Atro*. This might be due to VP16 toxicity during development, even though it was developed to be reduced. Indeed, no developmental problem was ever observed using both G*al4* and *LexA::GAD* when expressed with *polyQ-Atro*. It is also possible that *LexA::VP16* expresses a much higher level of *polyQ-Atro*, which becomes toxic during development. And the fact that it is Gal80 insensitive, it is then impossible to bypass the development of the fly. The *dEAAT1-LexA::GADs* expression levels may be weaker than *dEAAT1-Gal4*. To try and solve this issue I recombined both the *GAD1* and *GAD3* lines to increase the level of *polyQ-Atro* expression. However, the combination of the two did not increase the strength of the phenotype (**Figure 3.5B**). Actually, both control and *polyQ-Atro* flies have an identical lifespan. This is mostly due to the reduced controlled lifespan, caused possibly by a toxic effect of having two *LexA::GADs* expressed in the same cells.

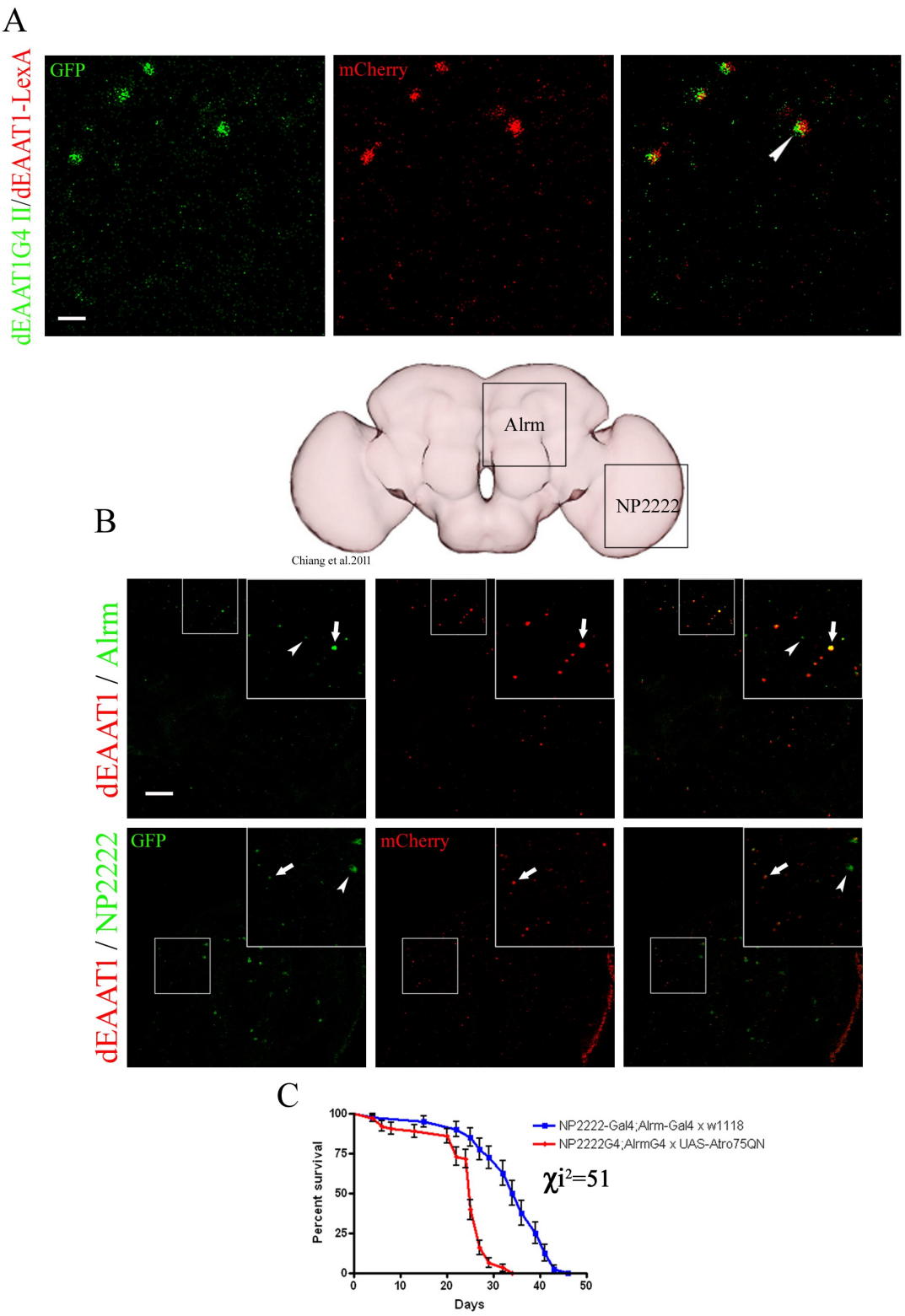


Figure 3.4: *Alrm-Gal4* and *NP2222-Gal4* partially colocalize with *dEAAT1-Gal4 II* and do not recapitulate its phenotype. Live confocal images of *Drosophila* adult brains. **(A)** Colocalization of cell nucleus between *dEAAT1-Gal4 II* and *dEAAT1-LexA* using *UAS-nls-GFP* and *LOT-mCherry* as reporters. Scale bar: 2μm. **(B)** Colocalization between either *dEAAT1-LexA* and *Alrm-Gal4* (top panels) or *dEAAT1-LexA* and *NP2222-Gal4* (bottom panels). Schematic above shows locations of the pictures. Scale bar: 20μm. Arrows indicate colocalized expression. Arrowheads indicate *dEAAT1-LexA* negative cells. **(C)** Lifespan of both *NP2222-Gal4* and *Alrm-Gal4* drivers crossed with either w^{1118} as control or *UAS-Atro75QN*. 60 flies are assessed in each set of experiment and the analysis was done using Prism GraphPad software with logrank test method. Error bars represent standard error.

dEAAT1-LexA::GAD1 was then used to identify which cell populations were targeted by the driver. Both *NP2222-Gal4* and *Alrm-Gal4* were combined with *UAS-nls-GFP*. Using the previously recombined *dEAAT1-LexA; LOT-mCherry* line, I looked at the colocalization profile for each of them. Most of the GFP dots from *NP2222-Gal4* and *Alrm-Gal4* colocalize with the mCherry from *dEAAT1*. However, some cells from each of *Gal4* drivers are not *dEAAT1* positive (**Figure 3.4B**). This result is surprising because the *dEAAT1* driver has been generally used to target astrocyte-like glia since it is from the glial glutamate transporter promoter and not cortex glia. However, from the CD8-GFP staining, some *dEAAT1* positive cells that had the same pattern as *NP2222* positive cells could already be observed (**Figure 3.2A and 3.2C**) as well as some ensheathing glia-like pattern in the optic lobe, surrounding axonal tracts. Altogether, these results mean that there is at least another glial population of astrocyte-like glia that does not express *dEAAT1*. Nevertheless, *dEAAT1* positive cells recapitulate a big part of *Alrm* and *NP2222* positive cells. Hence, I asked whether combining *Alrm-Gal4* and *NP2222-Gal4* drivers could phenocopy the *polyQ-Atro* lifespan phenotype of *dEAAT1-Gal4*. Surprisingly, expressing *polyQ-Atro* in both cell populations at the same time does not increase the phenotype obtained with *Alrm-Gal4* alone. Indeed, it has the same median survival for *polyQ-Atro* and a slightly reduced lifespan for the control, may be due to a toxic effect from the combination of both drivers (**Figure 3.4C**). Its Chi square is 50, which is in the range of both *Alrm-Gal4* and *NP2222-Gal4* Chi square values. It is possible that the effect of the impairment of both cell types together has reached a threshold of toxicity for the organism and that there is no additive effect possible in the shortening of the lifespan due to *polyQ-Atro* expression.

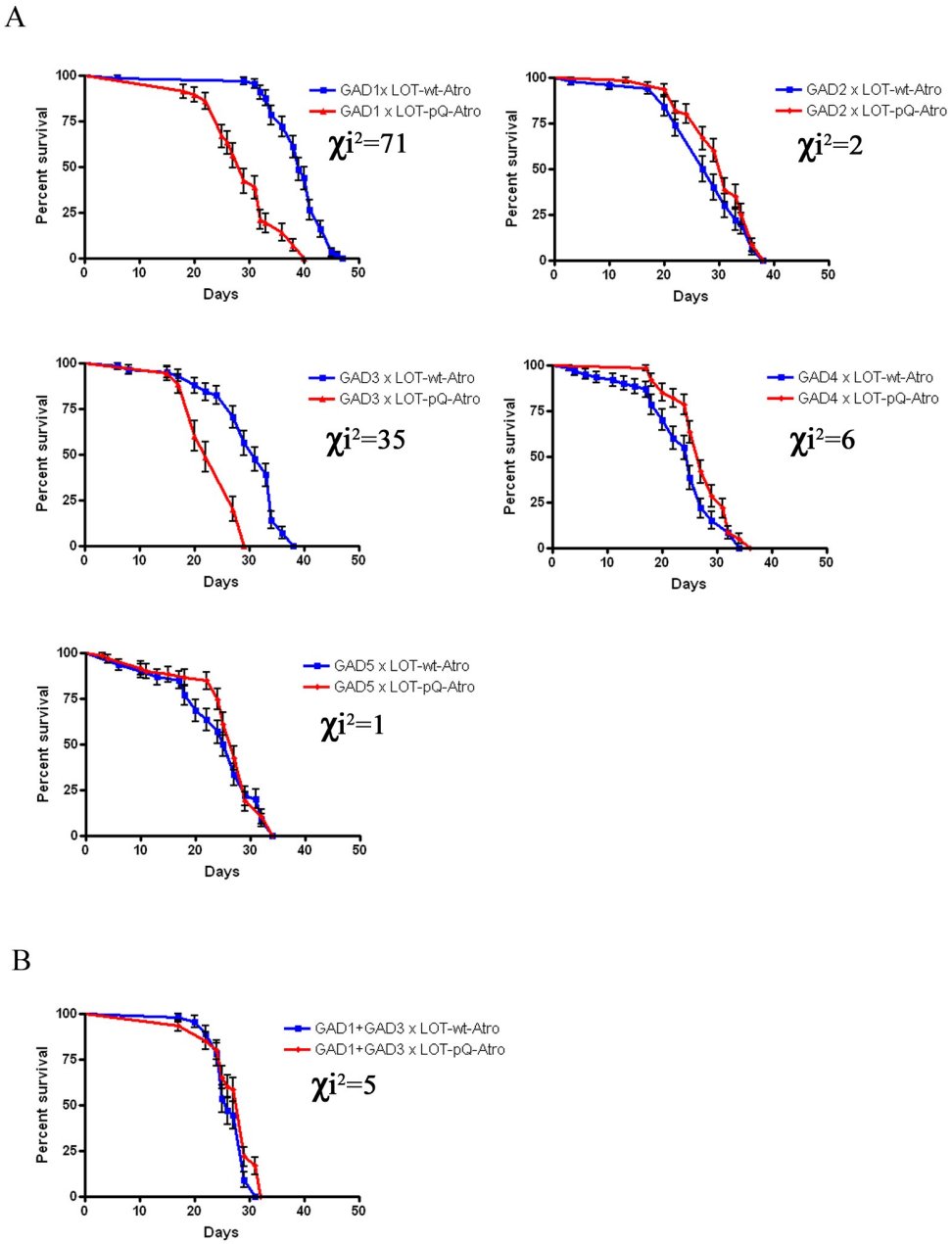


Figure 12: Lifespans of the different *dEAAT1-LexA::GAD* lines generated. Each lifespan is done at 29°C with 60 flies. Chi squares are given by Prism GraphPad software using the logrank test method. Error bars represent standard error. **(A)** Each of the 5 lines generated was crossed either to a wilt type *Atro* (*LOT-wt-Atro*) or a *polyQ Atro* (*LOT-pQ-Atro*) line. **(B)** *GAD1* and *GAD3* were combined on the same line and then crossed to either *wt Atro* or *polyQ Atro.*

It has been shown that *dEAAT1* could be expressed in some *repo* negative cells in the lamina of the adult optic lobe (Rival et al. 2004). To confirm this observation, *Repo-Gal80* was co-expressed beside *dEAAT1-Gal4>UAS-eGFP* to inhibit Gal4 in all glial cells. As expected, some cells remained labelled. The labelling of a group of neurons in the optic lobe expressing *dEAAT1* was confirmed as previously described; however, some cells and their processes were also seen around the central complex and the oesophagus (**Figure 3.6A**). However, the majority of the *dEAAT1* cells being glial cells, I wondered if it was possible to rescue the *polyQ-Atro* phenotype by the use of the pan-glial *Repo-Gal80*. The lifespan of *dEAAT1>polyQ-Atro* in the presence of *Repo-Gal80* is not fully rescued (**Figure 3.6B**) but the Chi square value decreases from 69 without *Repo-Gal80* to 16 with it, which is close to the variability level observed in these assays, between genetically identical controls, though statistically significant (both $p<0.0001$) (see next chapters). It is also possible that *Gal80* expression is not high enough to compensate the Gal4 expression by *dEAAT1* driver. I decided to try and rescue the *polyQ-Atro* phenotype of *Repo-Gal4* driver, which has a strong level of expression in all glial cells. As expected, *Repo-Gal80* expression was able to fully rescue the lifespan phenotype of *Repo-Gal4>polyQ-Atro* (**Figure 3.6C**). Indeed, the Chi square in this case goes from 106 without *Repo-Gal80* to 0.01 with it, which is not statistically significant. Thus, *Repo-Gal80* is capable of blocking the activity of *Gal4* expression to at least *Repo-Gal4* level. It is noteworthy that *Repo-Gal4* is a knock-in insertion of *Gal4* into the endogenous locus of the *repo* gene. However, *Repo-Gal80* is a construct made of a part of the *repo* promoter, and then inserted randomly into the genome, thus, potentially having a different level of expression. I then wondered if the fact that *Repo-Gal80* was not rescuing fully *dEAAT1* expression of *polyQ-Atro* was due to its expression in *repo*-negative cells and especially in the subset of *dEAAT1* positive neurons. This question was addressed by the use of *Elav-Gal80*, which similarly to *Repo-Gal80* is not temperature sensitive. The inhibition of any potential activity of Gal4 in neurons by *Elav-Gal80* did not rescue the lifespan phenotype (**Figure 3.6D**). Furthermore, compared to the respective lifespans without *Elav-Gal80*, it seems that this transgene is toxic and decreases mildly the *Drosophila* lifespan by itself. The Chi square is 96 with *Elav-Gal80*, and 67 without. However, from the same experiment done previously without *Elav-Gal80*, the Chi square of *dEAAT1-Gal4* II was in the range of 100. Overall, it suggests

that the neuronal cells positive for *dEAAT1* do not seem to have a major role in the reduction of the lifespan due to *polyQ-Atro* even though it is possible that the Gal80 level from the *Elav* promoter might not be sufficient to counteract the Gal4 level from the *dEAAT1* promoter. It is also possible that the driver's expression outside the CNS can impact the lifespan.

Taken together, these data show that *dEAAT1* positive glial cells are a mix of some astrocyte-like glia *Alrm* positive, some cortex glia *NP2222* positive and potentially some ensheathing glia in the optic lobe. However, the strong reduction of the lifespan by *dEAAT1* drivers was not recapitulated by combining *Alrm-Gal4* and *NP2222-Gal4* drivers. Furthermore, it seems that a subset of *Alrm* and *NP2222* positive cells do not express *dEAAT1-Gal4*. I also confirmed that the glutamate transporter driver is expressed in some neurons present in the adult optic lobe, but is also expressed in few neurons in the central brain. However, it is unlikely that its neuronal expression is responsible for the partial rescue observed with *Repo-Gal80*. It is possible that an expression in other tissue outside the CNS could have an effect on the lifespan, such as fat bodies and/or muscle.

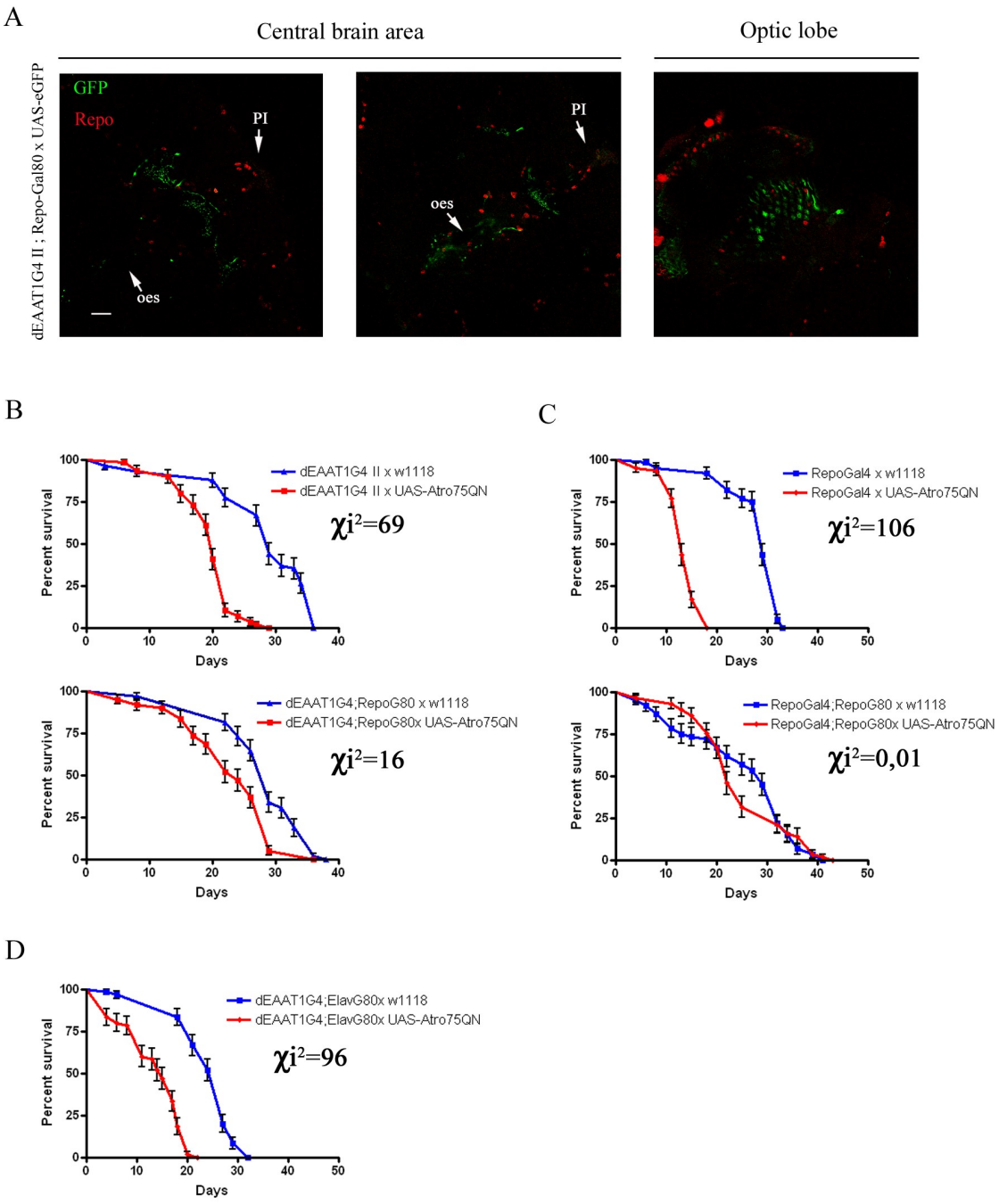


Figure 13: *dEAAT1* positive neurons expressing *poly-Atro* do not affect the lifespan. **(A)** Confocal images in different brain areas of cells expressing *dEAAT1-Gal4 II>UAS-CD8-GFP* but not *repo*. In red, *repo+* cells to not colocalize with GFP. PI: Pars Intercerebralis. oes: oesophagus. Scale bar is 10µm. **(B)** Lifespans of flies expressing *dEAAT1-Gal4 II > UAS-Atro75QN/w^{1118}* combined with *Repo-Gal80* (bottom) or without (top). **(C)** Lifespans using *Repo-Gal80* combined with *Repo-Gal4*, expressing *UAS-Atro75QN* or crossed with *w^{1118}* as control. **(D)** Effect of *Elav-Gal80* on lifespan of *dEAAT1-Gal4 II > UAS-Atro75QN/w^{1118}*. All lifespans have been done using 60 flies. Analysis was done as described in previous figures.

3.3 Discussion

3.3.1 Astrocytes and cortex glia are sensitive to *polyQ Atro*

Is there a certain variation of *polyQ Atro* toxicity among the different glial subtypes? Five types of glial cells present in *Drosophila* nervous system have been assessed. The outer layer of the BBB (the perineurial glia) has not been studied, as their role in the *Drosophila* CNS remains unclear. There is another kind of glial subtype recently discovered. These cells have been characterized as *Glutamine synthetase 2* (*Gs2*) and *dEAAT1* positive and are in very close proximity to the neuromuscular junctions (McCabe et al. 2015). *Gs2* is also present in wrapping glial cells, which form the BBB around the nerves in the peripheral nervous system. Using *Moody-Gal4* or *Nrv2-Gal4* drivers to downregulate *Gs2* in BBB/wrapping glia, *Gs2* was indeed knocked-down from those cells. However, some cells remained *Gs2* positive, having their cell bodies and processes closer to fine motor axon branches forming neuromuscular synapses. These cells seem to be peripheral astrocyte-like glia, but might be targeted by *dEAAT1-Gal4* driver. This discovery being just made, no specific *Gal4* drivers are yet available for this subtype.

The differences in the lifespan phenotypes from the different drivers assessed can come from different origins. First, the level of expression of each Gal4 driver has not been assessed since it is relatively difficult to compare the levels between different cell types and the immunofluorescence is not accurate to do so. Coming from different promoters, it is highly likely that the Gal4 expression levels are different and in a build-up process such as for polyQ aggregation it can change the onset of the phenotype. It is also possible that the temporal expression of the different lines is different, especially during development. Again, for the same reasons, if the expressions are not constant, it can affect the build-up process. Indeed, the expression of *polyQ-Atro* only during three days in adult does not affect the lifespan (data not shown). Also, it is possible that there is a developmental component in the lifespan phenotypes, which can differ from each driver by their developmental expression levels or the sensitivity of each cell type to the polyQ toxicity

during the different developmental stages. However, the use of Gal80 temperature sensitive later on in this thesis has shown that developmental defects do not account for polyQ-Atro toxicity using *Repo-Gal4* driver at least. This driver, having a strong expression level and covering most of the glial cell types during all *Drosophila* stages (apart from midline glia, not assessed here), it is likely that the other drivers specific to glial cells would not have a developmental component in their lifespan phenotypes.

In the case of *dEAAT1-Gal4* drivers, two different lines have been assessed, with similar strength of their phenotypes. The fact that they are on different chromosomes, and that they have a similar phenotype, means that problems of insertion toxicity or modulation of *Gal4* activity are less likely. The same applies for *Alrm-Gal4*, more specific to astrocyte-like glia, according to the mCD8-GFP stainings. The combination of both drivers from the II and III chromosomes did not increase the strength of the phenotype. *dEAAT1-Gal4* is also expressed in glial cells other than astrocyte-like glia. The labelling with mCD8-GFP revealed some cells surrounding neuronal cell bodies and/or axonal bundles, which seem to be cortex glia and/or ensheathing glia. The subperineurial glia would require more investigation to understand their role in the reduction of the lifespan. Both *Moody-Gal4* and *Gliotactin-Gal*4 overlap in expression pattern, being expressed in these "BBB" glial cells in the CNS and PNS. However, one is having a relatively strong effect, similar to *NP2222-Gal4* while the other one is having none. It is possible that the *Gliotactin* promoter used to trigger Gal4 expression is stronger than the *moody* promoter. Some co-staining would be needed to evaluate the overlap between both drivers. However, a LexA line would need to be established in order to carry out this investigation in the most rigorous way. Thus, it remains unclear whether subperineurial glia, or at least part of them, are sensitive to the toxic effect of *polyQ Atro* and participate to the phenotypes observed with *Repo-Gal4*. For the neuropil ensheathing glia, *MZ0709-Gal4*, unfortunately only one driver was available. In this case, the weak phenotype can be due to the weakness of the *Gal4* expression due to the insertion of the transgene. For the same reason, the phenotype observed for *NP2222-Gal4* (cortex glia) could be different using drivers inserted into other genomic locus. Also, the control lifespan for this driver is abnormally extended, similarly to *Gliotactin-Gal4*. Even though all *Gal4* drivers have been generated on a w^{1118} genetic background, over

time, mutations might have accumulated, which can result in phenotypic differences, especially relevant for the lifespan. To solve this problem, an isogenisation of all the lines used could be done on the same genetic background, with the experiment repeated straight after to avoid any accumulation of different mutations in each line. Another way would be to generate new drivers that are also specific to similar glial cell populations, or at least different insertions similarly to what was available for *Alrm-Gal4* or *dEAAT1-Gal4*.

However, the strongest phenotypes for specific glial subtypes are observed using drivers that target astrocyte-like glia and cortex glia (neuronal cell body ensheathing glia). *Gliotactin-Gal4* is also comparable to *NP2222-Gal4*, both of them having higher Chi square than *Alrm-Gal4* drivers but it is the only one having a phenotype for this subtype compared to the use of two for *dEAAT1-Gal4* or *Alrm-Gal4*. Taken together, these data strongly suggest that astrocyte-like glia are sensitive to *polyQ Atro*, and their dysfunction triggers a clear reduction of *Drosophila* viability. However, *dEAAT1-Gal4* does not seem to be very specific and I have been unable to pin down which other population of glial cells is responsible for the strong effect of *dEAAT1-Gal4*. Further investigation would be required to establish clearly the role of each glial subtype in the toxic effect of *polyQ Atro* on the *Drosophila* organism. The use of *Alrm-Gal80* combined with *dEAAT1-Gal4* for instance, would help to specifically isolate the role of the astrocyte-like glia population positive for *dEAAT1-Gal4* and understand their participation to the phenotype. Our results may suggest also that some glial cells would be more affected than others or that the dysfunction of some glial cells might have a higher impact on *Drosophila* viability than others. It is for instance still controversial whether BBB defects affect the lifespan of adult flies or not (Kim et al. 2010).

3.3.2 *dEAAT1-Gal4* expression is not restricted to a specific subset of glial cells

dEAAT1 is one of the two glutamate transporters expressed in the *Drosophila* nervous system, the other being *dEAAT2*. They are differentially expressed throughout development. In the adult, *dEAAT1* seems to be mostly glia specific, even though few

neurons in the optic lobe express it as well. However, *dEAAT2* is also neuronal and has actually a higher affinity for taurine and aspartate rather than glutamate (Besson et al. 2004, Besson et al. 2011). Being a glutamate transporter and expressed mostly in the neuropil (Rival et al. 2004), it was thought that *dEAAT1* would be an astrocyte-like marker. Different cell patterns could be observed by labelling these cells using the membrane-bound mCD8-GFP. Some star shape cells are supposedly astrocyte-like glia as expected, but neuronal cells bodies surrounded by GFP could also be seen, similarly to *NP2222-Gal4* (cortex glia) pattern. However, the use of Gal4 drivers using the promoter region of a gene do not necessary represent the gene endogenous expression and it is possible that *dEAAT1-Gal4* targets cell types that do not express the *dEAAT1* gene or vice-versa. Indeed, *flybase* website reports *dEAAT1* RNA expression in fat bodies, muscles and to a low level in the gut, all of them being able to affect the lifespan. I have demonstrated that using a newly established *dEAAT1* driver combined with *LexA*, the *dEAAT1* promoter does not only recapitulate astrocyte-like glia pattern in the CNS. It has a stronger phenotype than *Alrm-Gal4* with the expression of *UAS-polyQ-Atro*. However, expressing *polyQ-Atro* in both astrocyte-like glia and cortex glia at the same time does not match the strength of the *dEAAT1* lifespan phenotype. Furthermore, the expression of *dEAAT1-Gal4* in a subset of neurons does not seem to be responsible for the difference observed with the other drivers. It is also possible that the expression of *Gal4* in the *dEAAT1* cells is stronger than *Gal80* from *repo* or *elav* promoters. A leakage of *polyQ-Atro* expression due to a reduced but not fully inhibited *Gal4* activity could result in the partial rescue of the lifespan. It is also surprising that some *Alrm* positive cells do not express *dEAAT1-Gal4*. So far, nothing has been described on the potential heterogeneity of astrocyte-like glia in *Drosophila* but it is likely that there are different subtypes of astrocytes having specialized functions. The heterogeneity of astrocytes in mammals has been studied only recently and we are still far from understanding the real complexity of the functions achieved by the different types of astrocytes (Bayraktar et al. 2015, Bribián et al. 2015, Johnson et al. 2015, Schitine et al. 2015). It is likely that, there is also a certain diversity of astrocyte-like glia in *Drosophila*. *Drosophila* astrocytes are also known to express a GABA transporter called *Gat*, recently identified as astrocyte marker in the CNS (Muthukumar et al. 2014, Stork et al. 2014). The pattern of expression overlaps with the *Alrm-Gal4* one, being expressed exclusively in the

neuropil via the astrocyte processes. It is possible that some astrocytes have specialized in GABA clearance and do not express glutamate transporters.

The fact that *dEAAT1-LexA::GADs* are weaker than *dEAAT1-Gal4* drivers, and that *dEAAT1* promoter targets a rather complex population of cells, it makes its further use for the study of glial cell functions in the CNS not specific enough and thus not dissimilar from *Repo-LexA*, which is however stronger (see next chapter). Also, the expression of *polyQ-Atro* with *dEAAT1-LexA::VP16* drivers seem to be very toxic for the flies. This result is surprising as all the lifespan done so far, including *Repo-Gal4* and *dEAAT1-Gal4* with *polyQ-Atro*, had no developmental defects. Moreover, when *dEAAT1-LexA::VP16* is crossed with w^{1118}, adult flies are viable. Hence, the expression of *LexA::VP16* in *dEAAT1* positive cells is not toxic by itself. It is possible that the level of expression triggered by *VP16* is much higher than *Gal4* or *LexA::GAD* and thus, becomes toxic during developmental stages. In any case, the use of *dEAAT1-LexA* drivers generated by us do not appear to be of direct use for the study of *polyQ Atro* effects on adult glial cells.

Chapter IV:

Using a DRPLA fly model to screen for neuron-glia interactions

4.1 Introduction

As described in the previous chapter, *polyQ Atro* expression in glial cells display a dramatic phenotype, reducing lifespan and motility. Compared to the effects of *polyQ Atro* expression in neurons, glial cells seem to be more sensitive to its toxicity, pointing toward an important participation of glial cells in the pathology due to polyQ toxicity (Nisoli et al. 2010). Indeed, its glia specific expression triggers motor defects, which are attributable to neuronal dysfunctions. Also, it has been shown that there were no signs of cell death prior to organism death using the *dEAAT1-Gal4* or *Elav-Gal4* drivers (glial and neuronal drivers used in that study), suggesting that it is possible that the fly dies from general deregulation of CNS homeostasis before losing any cells. Altogether these results demonstrate that glia *polyQ Atro* expression triggers alterations in glia function, which may result in neuronal dysfunction through alterations in neuron-glia communication.

Beside the autonomous effect of *polyQ Atro* toxicity in different cellular pathways, it is also of interest to better understand how glial cells defects can impact neuronal functions. These two cell types interact at different levels, depending on their functions. As described in the previous chapter, different glia subtypes have an effect on viability when specifically expressing *polyQ Atro*. It suggests that neurons can be affected in many different ways, depending on whether the astrocyte-like glia are affected or cortex glia, which have different functions leading to different interactions with the neighbouring neurons. For instance, astrocyte-like glia dysfunctions can modify neuronal activity through changes in neurotransmitter uptake or synapse formation and stability for example (Kerr et al. 2014, Muthukumar et al. 2014). However, cortex glia, which surround neuronal cell bodies, are thought to provide trophic and metabolic supports for neurons. It is yet to be shown that the so-called lactate shuttle is also present in *Drosophila* but it has been recently demonstrated that glial glycolysis is essential for neuronal survival (Volkenhoff et al. 2015). Also, this cell type expresses the engulfment receptor *Draper*, which suggests that they could participate in the clearance of neuronal cell debris when needed (Doherty et al. 2009). Modifications in each of the glial functions interacting with neuronal pathways important for CNS homeostasis will affect organism viability. It is highly likely that the expression of

polyQ Atro will trigger large transcriptional alterations, leading to different functional changes in glial cells. The dysregulation of autophagy, which was reported also in glial cell (Nisoli et al. 2010), is also likely to affect the trafficking machinery and possibly also the secretory, phagocytic and endocytotic pathways, leading to the impairment in processes critical for several glial functions. However, relatively little is known about glia-neuron interactions, especially in a disease context. In most of the cases, models express the mutated protein in the entire CNS using prion protein promoters (*PrP*) for instance (Garden et al. 2002, Loftus et al. 2002, Shin et al. 2005), which prevent the specific understanding of the participation of only one cell type to the pathology. Or, when the study is restricted to one cell type, the mutated protein is most of the time expressed in neurons (Latouche et al. 2007, Thomas et al. 2010, Zhang et al. 2015), which is for historical reasons the most obvious target to tackle the impaired mechanisms of neuropathological disorders (Wong et al. 2002). However, increasing number of studies try to change the approach by specifically expressing mutated proteins in glia subtypes. Several have already demonstrated that it is sufficient to trigger neurodegeneration but so far much of the effort has been to characterize defects in neurotransmitter transport (Liévens et al. 2001, Custer et al. 2006) or in autophagy (Di Malta et al. 2012) for instance. Most of these studies have been done using mouse models, which limit the use of genetic modifications in order to modify pathways independently of the mutated protein expression. Thus, it is difficult to study neuron-glia interactions in a cell-type specific manner, which would help to decipher molecular pathways mediating the toxic effects from one cell to another.

Drosophila has been historically a model used to screen for genes (Dietzl et al. 2007) or compounds (Levine et al. 2016) that would modify a defined phenotype. Its costs, fast life-cycle and available genetic tools make it an ideal model to screen for a high number of selected genes in a reasonable time, which would not be possible in mice. A perfect example of this process is the recently published work using *C9orf72* screens on *Drosophila* that identified defects in nuclear-cytoplasmic transport, which has been then confirmed in iPSCs from patient and post mortem staining on ALS patient's brains (Freibaum et al. 2015, Tran et al. 2015, Zhang et al. 2015), highlighting the relevance of such screens for the discovery of disease mechanisms conserved across species. In order to

uncover new interactions between neurons and glia that could be involved in mediating glial polyQ toxicity, several screens have been designed in our lab. Using the broad range of genetic tools available in *Drosophila*, it is possible to express independently two transgenes in two different cell types.

In this study, the focus is on the role of impaired glial cells in neuronal dysfunction. It has been established that expressing *polyQ Atro* in glial cells reduces *Drosophila* lifespan. Using this paradigm, the aim of this chapter is to discover genes expressed in neurons that would be involved in the mediation of the glial polyQ toxicity on the lifespan. Using this strategy, it would be possible to uncover neuronal genes that are required to detect phenotype of glial impairment. Indeed, the effect of polyQ expression in glial cells is unclear and the impairment of the glia-neuron communications in polyQ diseases remain unknown.

4.2 Results

4.2.1 Establishment of a new DRPLA fly model to study glia-neuron interactions

In the previous chapter, the characterization of the impact of each glia subtype was done using a full length transgenic *polyQ Atro* and was expressed throughout development that has no or very little impact on viability or on lifespan. In order to identify genes expressed in neurons that modify the adult lifespan, an RNAi-based strategy was chosen to knock-down specific genes. For the *Drosophila melanogaster*, more than 80% of the genes have their corresponding RNAi lines. Also, 3 to 4 different RNAi collections exists from different stock centers (VDRC "*stockcenter.vdrc.at"* (Austria) and Bloomington "*flystocks.bio.indiana.edu"* (USA) being the main ones), each of them having generated sometimes several UAS-IR (inverted repeats) RNAi lines per gene, which allow the confirmation of a phenotype by independent lines binding other sequences of the mRNAs. However, the downregulation of genes in neurons through development is often lethal or could trigger developmental defects independently of their adult function (Koizumi et al. 2007, Paradis et al. 2007, Valakh et al. 2012). To by-pass this problem, a ubiquitously expressed Gal80 temperature sensitive transgene will be used (*Ubi-Gal80^{ts}*). This temperature dependent Gal4 inhibitor allows the control of the transgene activation, by transferring the flies from 18°C (Gal80 active) to 29°C (Gal80 inactive). Thus, it will be possible to specifically activate transgene expressions only at the adult stage. The impact of the gene knock-down on the lifespan will then come from adult function and not developmental modifications, which could have later impact on the lifespan of the fly.

Being a non-cell autonomous screen, the transgenes have to be expressed in different cell types. Two different expression systems are used to independently express *polyQ Atro* in glial cells and the RNAi in neurons. The RNAi collections available are all UAS- based lines, meaning that they are activated by Gal4. Thus the *Elav-Gal4* driver will be used in order to express the different RNAi lines in all post-mitotic neurons. There are other

expression systems available in Drosophila, like the Q-system, which is drug dependent. However, most of the tools available beside the Gal4-UAS system have been developed for the LexA-LexAop (LOT) system, where a *Repo-LexA* line is actually already available. Moreover, even though LexA binds specifically to LOT promoter sequence and do not cross react with UAS, it is still Gal80 sensitive. Indeed, the LexA drivers have been developed mostly with the activating domain of Gal4 and the DNA binding domain of LexA. The combination of both systems will allow the activation of both transgenes at the same time, at the adult stage in this case.

The aim of this screen is to identify pathways that are impaired by the expression of a disease protein. However, the *Drosophila* homolog of *Atrophin-1*, called *Atro*, is slightly different from the mammalian version, as described in the first chapter. The Drosophila *Atro* possesses some domains shared with the human ortholog *Atrophin-2*, which is not responsible for the disease DRPLA (**Figure 4.1A**). However, *Atro* is known to give rise to two different isoforms, recapitulating both *Atrophin-2* and *Atrophin-1* expression in mammals (Fanto, unpublished). The overexpression of the full length *Atro* would correspond to the overexpression of both Atrophin genes expressing a polyQ mutation in human. It is possible that the overexpression of a protein containing such domains, present in both *Atrophin-2* and *Atro* but not *Atrophin-1*, could have undesired molecular changes irrelevant to what is happening in DRPLA. To avoid such potential issues, and build a more faithful model for DRPLA, a shorter version of *Atro* lacking the N-terminal domains has been designed, downstream of a LOT- sequence, called *sAtro*. This short version of *Atro* has been constructed with either a wild type stretch of 11 CAG repeats or a disease length stretch similar to the UAS- used in the chapter III, containing 75 CAG repeats (**Figure 4.1A**). The polyQ construct (*LOT-polyQ-sAtro*) will allow the overexpression of only the Atro isoform lacking the N-terminal domains thus not interfering directly with the functions of these domains and resembling more closely the functions of *Atrophin-1* in humans. This new construct will help modelling more closely DRPLA glial cells. A non-polyQ construct (*LOT-wt-sAtro*) has been designed in order to control the effect of the overexpression of *Atro* independently of its polyQ stretch. It will allow us to determine whether the phenotype is polyQ dependent or due to the overexpression of *Atro* itself.

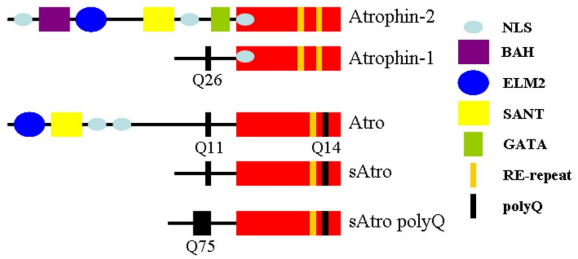
The plasmids containing the different versions of *LOT-sAtro* (pQ or wt) were sent to be injected at Fly-Facility in Clermont-Ferrand. Around a hundred and fifty embryos have been injected for each plasmid. The *Atro* expression for each of the positive insertion was confirmed first by immunostaining using this time *Repo-LexA* to visualize the presence of overexpressed *Atro* in glial cells in the L3 larval eye imaginal discs. Two *LOT-polyQ-sAtro* lines were positive, named *A* and *B* (**Figure 4.1B**). The same method was applied to generate two *LOT-wt-sAtro* lines, one on the second chromosome and one on the third. Both *A* and *B* lines being inserted on the second chromosome, a *LOT-wt-sAtro* line on the second chromosome was selected as well for later use, in order to simplify crossing schemes.

The first step was then to generate a line in which there would be both drivers (*Elav-Gal4* and *Repo-LexA*), glial *polyQ Atro* (*LOT-sAtro-polyQ*) and a ubiquitous Gal80 temperature sensitive (*Ubi-Gal80^{ts}*) transgenes and then characterize the lifespan of this line regarding a potential toxicity due to the presence of all these transgenes together in the same organism. The *LOT-sAtro-polyQ* lines have been recombined with *Ubi-Gal80^{ts}* and then combined with the two drivers present on the X chromosome. The final genotype of the line later used for the screen becomes: *Elav-Gal4*, *Repo-LexA* (X chr); *LOT-sAtro-polyQ*, *Ubi-Gal80^{ts}* (II chr). All these transgenes are homozygous viable. The toxicity of both polyQ lines was then evaluated by lifespan in order to see if the combination of *Repo-LexA* with this new *polyQ sAtro* line would give a similar phenotype to *Repo-Gal4 > UAS-Atro-polyQ*. Both lines were crossed to *w^{1118}* flies. The toxicity of the drivers alone or the transgenic recombined lines without driver was assessed. The lifespan of both final lines (with the drivers) have identical median survival of 17 days, very similar to what was obtained in the chapter III with *Repo-Gal4* and without Gal80^{ts}, which was 15 days. This difference of 2 days can come from the strength of *Repo-LexA* driver compared to *Repo-Gal4* or the efficiency of the LOT compared to UAS as well, or an intrinsic difference between *Atro* and *sAtro* toxicity. However, when the lines without the drivers are crossed with *w^{1118}* flies to assess a potential toxic effect of the insertion itself, there is a difference between the two transgenic lines. While *A* lifespan is similar to the drivers alone, with a median survival of 33 days, the

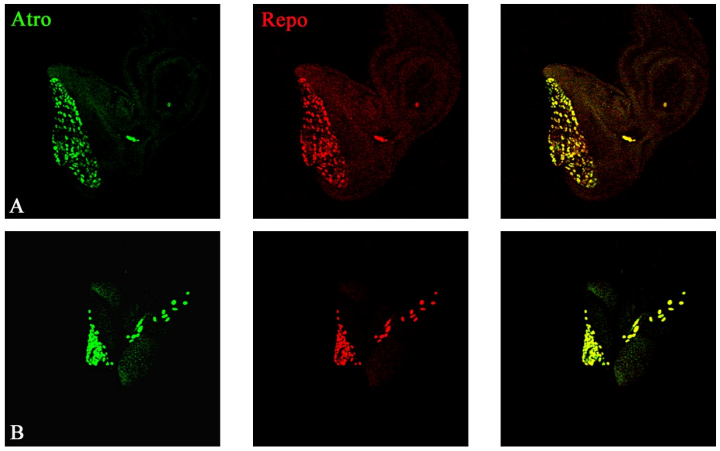
B line has a median survival of 26 days (**Figure 4.1C**). This difference suggests that the transgenic insertion of the *B* line is somewhat toxic for the organism, differently from the *A* one. The respective Chi square for each line compared to their own control are 86 and 75. Those numbers, compared to the 111 obtained in the chapter III confirms that the phenotype is slightly weaker but still in the range of a pan-glial effect of *polyQ Atro* toxicity on the *Drosophila* organism.

The *A* line being the intrinsically less toxic of the two has been the one chosen for the screen.

A



B



C

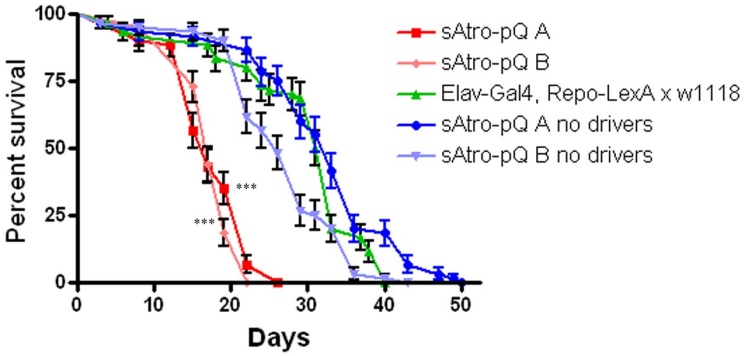


Figure 4.1: Characterization of the lines generated for the screen. (A) Comparison between human and *Drosophila* Atrophins/Atro proteins. **(B)** Stainings of L3 larvae eye imaginal discs with anti-Atro and anti-Repo on lines expressing *LOT-sAtro-pQ* lines using *Repo-LexA* as driver. A and B are positive for Atro in glial cells. **(C)** Lifespans for both positive lines recombined with UbiGal80ts and crossed with *Elav-Gal4, Repo-LexA* drivers or w^{1118} as a control without expression of the transgenes. *sAtro-pQ A/B: Elav-Gal4,Repo-LexA; UbiGal80ts,LOT-sAtro-pQ A/B*. When "no drivers": *UbiGal80ts,LOT-sAtro-pQ A/B x w1118*. 60 flies are assessed per genotype. The statistical analysis was done as previously described. ***: $p < 0.0001$

4.2.2 Identification of glia-neuron interacting genes using an RNAi-based screen

4.2.2.1 Selection criteria for the screening

The first criterion to define was the size of the screen. How many genes to be screened? A reasonable number of genes decided to be screened by RNAi was around a thousand, which corresponds to less than 10% of the *Drosophila* genome (between 14000 and 18000 genes located to the genome). Each gene would be downregulated by only one RNAi line as a compromise between number of gene screened and reliability on the efficiency of the RNAi. By setting up on average 35 crosses per week, it should be theoretically possible to screen this amount within 8 months since it takes around two months from the set up of the cross to the end of the lifespan of the progeny. The VDRC collection being the most complete one and made on w^{1118} genetic background (compare to the Bloomington one made on *y*,*v* background), all the RNAi lines were ordered from them. The most recent RNAi designed by the consortium is the so called KK line. Contrary to its former version called GD, the KK is site-directed inserted into the second chromosome, to a location known to allow high transgene expression. The GD lines are randomly inserted and can be present on either the second or the third chromosome. All the RNAi lines ordered were KK lines or GD only when the KK were not available.

The second criterion chosen was the enrichment of the gene expression in the head against the rest of the body. Using the website *Flyatlas.org*, it is possible to sort the genes by a multiple range of characteristics. The expression profile of most of the genes has been characterized allowing the selection of specific expression patterns (Duncan et al. manuscript in preparation). The aim of this study is to find new genes involved in the communication between glial cells and neurons and thus, it is more likely that genes involved in the interaction between those two nervous system specific cell-types are more enriched in the brain compared to the rest of the body. It is obviously possible that very common molecular mechanisms, such as autophagy, cell death or cell cycle genes will be

disregarded using this method but it will allow us to focus on genes more specific to the nervous system and thus more likely to be involved in CNS specific functions. A third criterion for the selection was the availability of overexpression lines. Then, from this list, the first thousand genes matching these criteria were chosen.

4.2.2.2 Screening method and results

From the experience the lab has acquired in doing lifespan, a number of 60 flies (3 tubes of 20 flies) per condition was chosen. This number of flies is a compromise between the time spent to count dead or alive flies three times per week and having a sample size big enough to detect differences in lifespans. As mentioned in the previous chapter, the Chi square value given by the software used for the statistical analysis (Prism GraphPad) is the number used to compare different strength of phenotypes. This number, related to the P-value, is more intuitive in its use than the P-value itself since it is directly proportional to the strength of the phenotype. However, likewise, it is also linked to the number of specimen assessed in each condition meaning that it can only be used for the comparison of sets of experiments using the same number of flies. It has already been established in the lab that in the condition of 60 flies assessed per lifespan, a Chi square smaller than 10 is not reliable and often not reproducible even though it is transformed in significant P-value. Indeed, two genetically identical control lifespans with this amount of flies can have a Chi square of 10, giving a statistically significant P-value, which represent the variability of the assay in these conditions. Another reason for choosing to use the Chi square rather than the P-value, is that we use this parameter only for quantification and ranking of phenotypes and we do not wish to assume any statistical or biological threshold of significance in our analysis because of the intrinsic variability of the lifespan assay. Moreover, above a Chi square value of 20, the software does not give the exact value of the P-value, just mentioning $p<0.0001$, preventing any possibilities to rank the strength of the phenotypes. The aim with this reasoning is to pick up the most reliable candidates, with a statistically significant P-value but also with a higher threshold of selection using a Chi square value above 10, which represent a P-value of 0.01.

Out of the thousand RNAi lines assessed by lifespan, 93 of them have been identified to be potential suppressor (increase of lifespan) or enhancer (decrease even further the lifespan). The decision to select those candidates was however taken by the evaluation of the female lifespan. This modification of our intentions was made necessary because male lifespan was barely variable and very few RNAi lines (<5) sensibly modulated their lifespan, masking any potential effect. This represented a caveat for the screen, which became evident only after 30% of the crosses had been already set up and the first ~100 lifespan were analysed. Although this reduced the sample size of each lifespan experiment it was decided to carry on with the initial set up not to invalidate comparisons with the part of the screen already done and because the lifespan of 30 fertilised females seemed to deliver meaningful information at that point. In hindsight, it is possible that the use of drivers on the X chromosome, present in only one copy on the males, has impacted on the lifespan. The genes present on the X chromosome of the males are known to be more expressed than on the female X chromosome, having two alleles. Indeed, the final genotype of the assessed flies was: *Repo-LexA,Elav-Gal4*/(wt X or Y from the UAS-IR line) ; *UbiGal80^{ts},LOT-sAtro-pQ/UAS-IR*. This could lead to a stronger expression of *polyQ Atro* in males that could limit the observation of phenotypes.

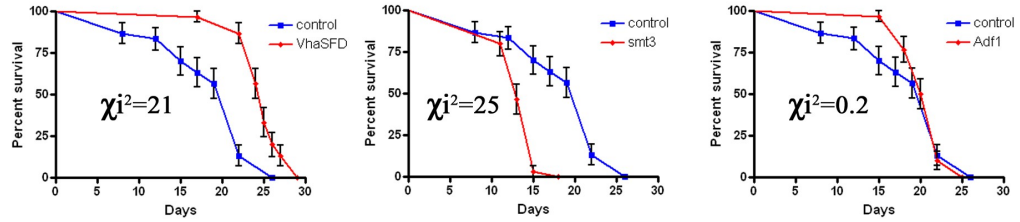
The candidate that had the strongest suppressor effect in females is *VhaSFD* RNAi, with a Chi square of 21 compared to a control done at the same time with the same number of flies. The one with the strongest enhancer effect is *smt3* RNAi, with a Chi square of 25 (**Figure 4.2A**). As an example, the lifespan of an RNAi line (*Adf1*) without phenotype is also shown in **Figure 4.2A**, having a Chi square value of 0.2. These values correspond to the analysis of 30 flies (females only).

Then, these 93 lines have been repeated all at once in order to confirm the phenotypes observed in the first screen, especially since only 30 flies were analysed. Beside the RNAi lines several controls were run. This allowed the generation of three random pairs of controls, each pair combining two control lifespans, in order to get the same number of flies compared to each repeated line, which will rich 60 females assessed. These three pairs have been compared to each other in order to evaluate the variability of the assay in these

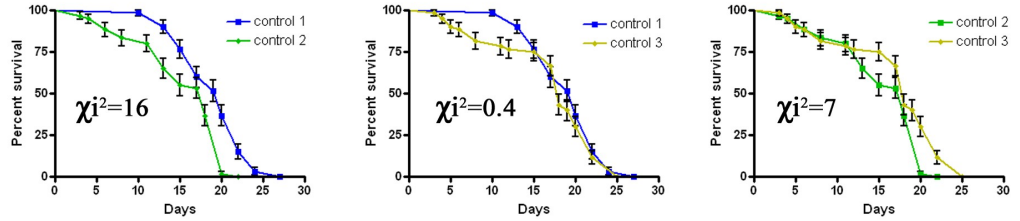
conditions. The Chi square given by the comparison to each other ranges from 0.4 to 16 (**Figure 4.2B**). On average, the difference between control lifespan gives a Chi square of 7.8, which gives the threshold of confidence for this method of analysis, using 60 females and 3 pairs of controls.

Each of the repeated line was then compared to each of the established control as shown in **Figure 4.2C** for *VhaSFD* RNAi line. Having doubled the number of flies assessed this time, with 60 females, the Chi square doubles from the first screen since this value is dependent on the number of flies assessed. For a similar effect in term of lifespan, using double the amount of flies has roughly doubled the Chi square value as seen with the first and third graphs in **Figure 4.2C**, going from 21 with 30 flies in **Figure 4.2A** to 45-53 with 60 in **Figure 4.2C**. The three Chi square values were then averaged and the repeated genes were ranked according to their strength. The table in **Figure 4.2D** shows the repeated lines that have an average Chi square value bigger than 7.8, being the limit of the assay. 17 RNAi lines have a suppressor effect, rescuing the lifespan from *polyQ Atro* expression in glia and 12 have enhancer effect, decreasing even further the lifespan. The genes having an effect have a broad range of functions such as zinc transport for *ZnT77C*, acidification of vacuoles (*VhaSFD*), axon guidance (*caps*) or sumoylation (*smt3*). Some of them do not have any known function yet or are only predicted to have some according to their sequence homology.

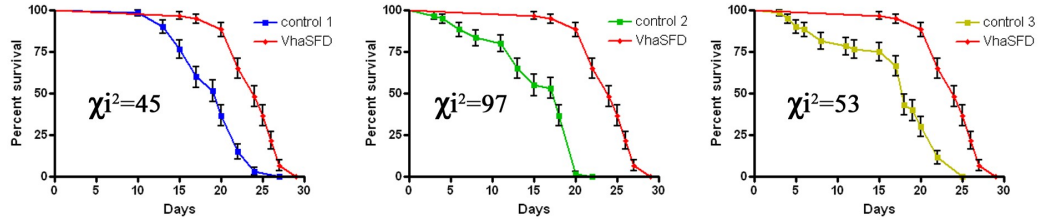
A



B



C



D

Suppressor Genes	Chi Square		Enhancer Genes	Chi square
VhaSFD	65.2		smt3	43
ZnT77C	63.3		lama	22.53
Idgf4	55.5		ArfGAP3	17.3
pum	55		lh	17.18
MKP4	47.98		CG12054	17.05
caps	45.96		SKIP	15.18
CG31534	40.33		Tsp42Ef	13.05
AnxB10	37.57		Bsg	13
CG2926	32.52		CG31729	12.59
CG4538	28.9		*lace*	12.17
EcR	27.6		CG12163	10.86
Reph	26.7		RpLP0	9.51
dac	21.8			
Plod	19.6			
CG10512	19.18			
mfas	18.9			
CG11138	18.5			

Figure 4.2: Examples of lifespans and method for the analysis of the non-cell autonomous RNAi screen. (A) Examples of lifespans from the first screen. Each of them represent 30 females for each genotype as the males did not have any phenotype in most of the cases. w^{1118} is used as control. Either the control or the RNAi against the named gene is crossed to *Elav-Gal4,Repo-LexA; UbiGal80^{ts},LOT-sAtro-pQ*. The Chi square value was used to rank the potential candidates to be repeated. **(B)** Lifespans of three sets of two controls combined together to evaluate the variability of the assay using 60 female flies. Each of the repeated candidate would be compared to each of these three sets of control to be ranked. **(C)** Example of a suppressor compared to each of the control set. The three Chi square obtained are averaged. **(D)** List of all the RNAi gene suppressors and enhancers which have an average Chi square value higher than the average of the controls. In orange, candidate genes being non-specific to *polyQ Atro* (see **Figure 4.3C**).

4.2.2.3 Validation of the specificity of the target genes

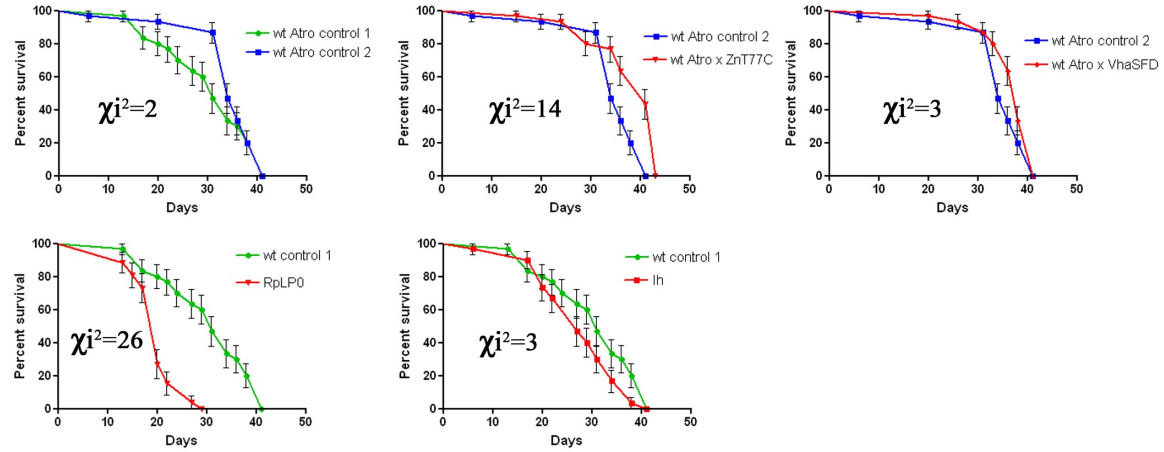
The RNAi lines that have an effect on the lifespan could have an effect independently from the toxicity of *polyQ Atro*. In order to distinguish the interaction between the downregulated genes with glia impairment from intrinsic effects of the RNAi line polyQ independent, the *wt-sAtro* line previously generated was used instead of the polyQ one. Two different controls have been obtained using this line. Their Chi square values when compared with each other is 2 (**Figure 4.3A**). For this validation, only two controls have been generated. Thus, the pair-averaging method previously done cannot be used for the analysis. However, with 30 flies analysed, any Chi square value below 10 will not be considered biologically relevant. More flies would need to be analysed in order to determine a higher confidence in a potential interaction. All the interacting gene RNAi lines have been compared to the controls. For the suppressors, only the RNAi lines against the zinc transporter (*ZnT77C*) and *idgf4* show an increase of the lifespan, with a Chi square value of 14 and 10.4 respectively, which is still on the low side of confidence (**Figure 4.3A**). *VhaSFD* RNAi line, which was the strongest suppressor, does not increase further the lifespan when *wt-Atro* is expressed in glial cells, indicating that the previous effect observed was specific to *polyQ Atro*. For the enhancer genes, 3 RNAi lines decrease the lifespan when *wt-Atro* is expressed in glial cells, *CG31729*, *lace* and *RpLP0* (**Figure 4.3A and 4.3C**). It is possible that the downregulation of these genes interact with *wt Atro* even though this does not trigger any lifespan phenotype by itself. It has actually been shown previously in the lab that the *UAS-wt-Atro* line was expressed to a higher level than *UAS-polyQ-Atro* without having any lifespan phenotype, ruling out a potential detrimental effect from the overexpression of the Atro protein itself. However, this control has not been done with the *LOT-wt/polyQ-sAtro* lines. To rule out a possible interaction with *wt-sAtro*, the RNAi lines having an effect in this case would need to be assessed without the expression of any *Atro* in glial cells. Most likely, however, the downregulated genes have an important function in neurons and are necessary for the CNS homeostasis independently of any *Atro* expression. It is actually rather surprising that such a very low number of RNAi lines decreased the lifespan when expressed in neurons in the screen, as this was the category of

false positive that was the most expected. The use of glial *wt-sAtro* indicates that most of the effects observed in the screen come from the interactions of the genes with *polyQ* toxicity and not the overexpression of *Atro* itself or the intrinsic function of the different RNAi in neurons. However, it is also possible that some of the effects are due to off-target effects and that the interacting genes are not the ones supposedly targeted by the RNAi. Even though the websites of the stock centers provide potential off-target and in most cases there is none, it cannot be ruled out.

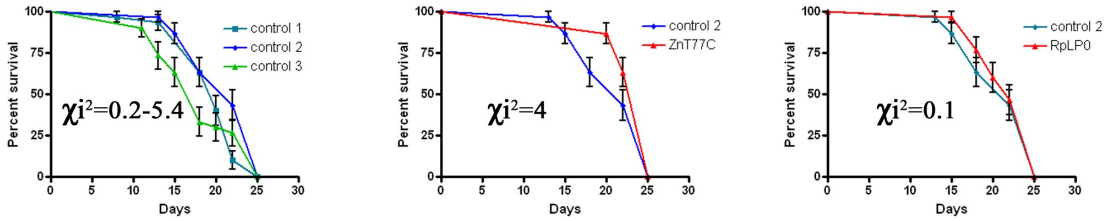
Another possibility would be that the insertion of the RNAi line has an effect in itself, by disrupting the expression of neighbouring/affected genes. Thus the phenotype would come from the affected genes and not the RNAi. Thus, a line expressing *LOT-polyQ-sAtro* was generated in the same condition as before but without *Elav-Gal4* to prevent the expression of the RNAi in neurons and thus, assess its potential transgene insertion effect. None of the 5 RNAi lines assessed had any phenotype, indicating that the insertion of the transgenes does not affect the lifespan (**Figure 4.3C**, lower table). Thus, the phenotypes of these 5 RNAi lines in *wt-Atro* condition are likely to come from an intrinsic effect of the downregulation of these genes in neurons or reflect a possible interaction with the overexpression of *wt-Atro* and are highlighted in orange in each of the previous tables to show their positions in the ranking of the candidate genes.

Given the diversity of the positive candidate gene functions, I decided to focus on transmembrane proteins since such molecules are natural candidates for mediating direct cell-cell interactions. Indeed, several candidate genes are involved in cell adhesion and some of them, like *capricious* (*caps*), are already known to be involved in neuron-neuron or neuron-muscle interactions or *midline Fasciclin* (*mfas*), involved in neuron-neuron/glia interaction.

A



B



C

Wild-Type Atro Lifespan				
Suppressor Genes	**Chi Square**		**Enhancer Genes**	**Chi square**
VhaSFD	3		smt3	6.7
ZnT77C	14		lama	1
ldgf4	10.4		ArfGAP3	0.1
pum	4.5		lh	3
MKP4	0.1		CG12054	0.001
caps	5		SKIP	2
CG31534	2.3		Tsp42Ef	8.4
AnxB10	0.02		Bsg	1.3
CG2926	0.1		CG31729	17.8
CG4538	5.5		lace	12.8
EcR	3.2		CG12163	4.6
Reph	7.2		RpLP0	26
dac	2			
Plod	2.6			
CG10512	0.6			
mfas	1			
CG11138	5			

No Elav-Gal4 - RepoLexA > pQ-Atro Lifespan				
Suppressor Genes	**Chi Square**		**Enhancer Genes**	**Chi square**
ZnT77C	3.9		lace	5.7
ldgf4	1.9		CG31729	4.6
			RpLP0	0.1

Figure 4.3: Validation of the potential candidates expressing wild-type *Atro* in glia or no RNAi expression in polyQ conditions. (A) Examples of lifespans with wild-type *Atro* for some suppressor and enhancer candidate RNAi lines. The graph on the top left represents the difference between two extreme control lifespans. The genotypes are: *Elav-Gal4,Repo-LexA;UbiGal80^{ts},LOT-sAtro-wt* crossed to either *w^{1118}* or the RNAi of the named gene. **(B)** Examples of lifespans done without *Elav-Gal4*. The RNAi is not expressed in order to assess the effect of the transgene insertion for a potential interaction with wild-type *Atro* previously assessed. The genotypes are: *Repo-LexA;UbiGal80^{ts},LOT-sAtro-pQ* crossed either to *w^{1118}* or the RNAi of the named gene. **(C)** Summary of all the RNAi lines assessed for each of the control set up. Only the lines with previous Chi square superior to 10 were assessed for the next control step consisting in the absence of RNAi expression in polyQ conditions. In orange, candidate genes non-specific for *polyQ Atro*. All lifespans and analysis were done as previously described.

4.2.3 *mfas*, *caps* and *trn*: potential candidate genes interacting with glial *polyQ-Atro*

4.2.3.1 Effects of *mfas* downregulation in glial cells and neurons in the glial DRPLA model

mfas has been named from its expression pattern during embryogenesis where it is expressed in the CNS midline cells and trachea and is a member of the Fasciclin-I related family (Hu et al. 1998). However, no further studies have since come out apart from one where it is suggested that *mfas* could increase the haematopoietic tumour formation from a *hop* mutant background (Bina et al. 2010). In the RNAi screen, the downregulation of *mfas* in neurons in combination with the glial *polyQ Atro* expression rescued the lifespan even though the effect was mild, being one of the weakest phenotype among the suppressor genes. However, out of the 29 confirmed interacting genes from the RNAi screen, *mfas* is the only one also predicted to have a similar effect from the miRNA screens performed in our lab (see also next chapter). Interestingly, the analysis of the miRNA screens indicates that its downregulation in glial cells in co-expression with *polyQ Atro* would decrease even further the lifespan, thus, having an opposite effect in glia and in neurons. To confirm this prediction, several RNAi lines against *mfas* were assessed in glia *polyQ Atro* condition. Both RNAi lines, KK and GD, worsen the *polyQ Atro* phenotype, confirming the miRNA prediction (**Figure 4.4A**). However, another line was assessed, from the Bloomington collection, which is in a different background. Thus, as a control, an RNAi against mCherry on a TRiP background was used. Surprisingly, *mfas* knock-down using this line rescued the lifespan. *mfas* is a very complex gene having at least 16 predicted isoforms. It is possible that the RNAi lines used target different isoforms and thus could have different effect. However, even though they target different exons, the KK and GD lines target the exon 8 and the TRiP one targets the 6th exon, both exons are present in all isoforms. Thus it is unlikely that the different RNAi lines target different isoforms and should downregulate the expression of all of them. Thus, it is unclear what the real effect of downregulating *mfas* is in the DRPLA fly model. At the same time, the new RNAi lines (GD and TRiP) were tested

in neurons to confirm the phenotype obtained with the KK line in the RNAi screen (**Figure 4.4B**, left panel). Both of them extended weakly the lifespan, similarly to the KK line. Their Chi square values are 8 for the GD and 20 for the TRiP (**Figure 4.4B**). Thus, downregulation of *mfas* in neurons extends weakly but reproducibly the lifespan of DRPLA flies.

As a next step we decided to isogenise the *mfas* RNAi lines from the VDRC collection in an isogenic w^{1118}. Isogenisation is known to reduce genetic variability and is commonly used in lifespan assays as definitive proof of any given effect. Each line has been backcrossed 6 times in this w^{1118} strain. The lifespan have been then repeated using these newly established lines. Using 60 to 90 females for the lifespan, the GD line shows a significant increase in the lifespan with a Chi square of 46 (**Figure 4.4B**). Unfortunately, because of a technical issue not enough flies were obtained to analyse the lifespan of the KK line at this time. Thus, this experiment has to be repeated. However, this result with the GD line suggests that for weak phenotypes, the diversion of the genetic background over time can have a detrimental effect on the lifespan and hide potential effects. The TRiP line is on a different genetic background (*y*, *v*), and thus would require a different isogenisation protocol, which I have not performed for lack of time.

It is rather surprising that the TRiP line has an opposite effect in glial cells compared to the KK and GD lines while it has a similar phenotype when expressed in neurons. Because *mfas* has not been investigated and no antibodies have been generated, it is not possible to assess the efficiency of the different RNAi lines. In order to go further in the investigation of its potential function, I tried to generate a genomic mutation in *mfas* using the P-element jump-out technique. Indeed, there is a P- element (transposable element randomly inserted into the genome to overexpress or mutate genes) inserted into the promoter region of the gene. It is possible to remove inserted P-elements by the use of a line that expresses an enzyme called *transposase* that can "jump out" the element and normally allows the recombination back of the chromosome in a correct reading frame. However, sometimes, a correct recombination fails and a big piece of chromosomal DNA can be removed together with the P-element, leaving behind a deletion for the gene in which the P-element is

inserted. However, among all the 130 lines jumped out for *mfas*, none of them removed *mfas*. Indeed, some PCR primers were designed on the first exon, next to the P-element insertion, but all homozygous knock-out embryos were positive for the amplified fragment by PCR (data not shown). It means that it is very likely that all the lines generated have removed correctly the P-element from their genome. In order to understand *mfas* function in adult, which could be different from development, a knock-in/out by the CRISP/Cas9 method would need to be done. It would allow the possibility to tag the endogenous gene and knock it out specifically in the adult. However, this method would require months of work to establish the line before being able to investigate its function.

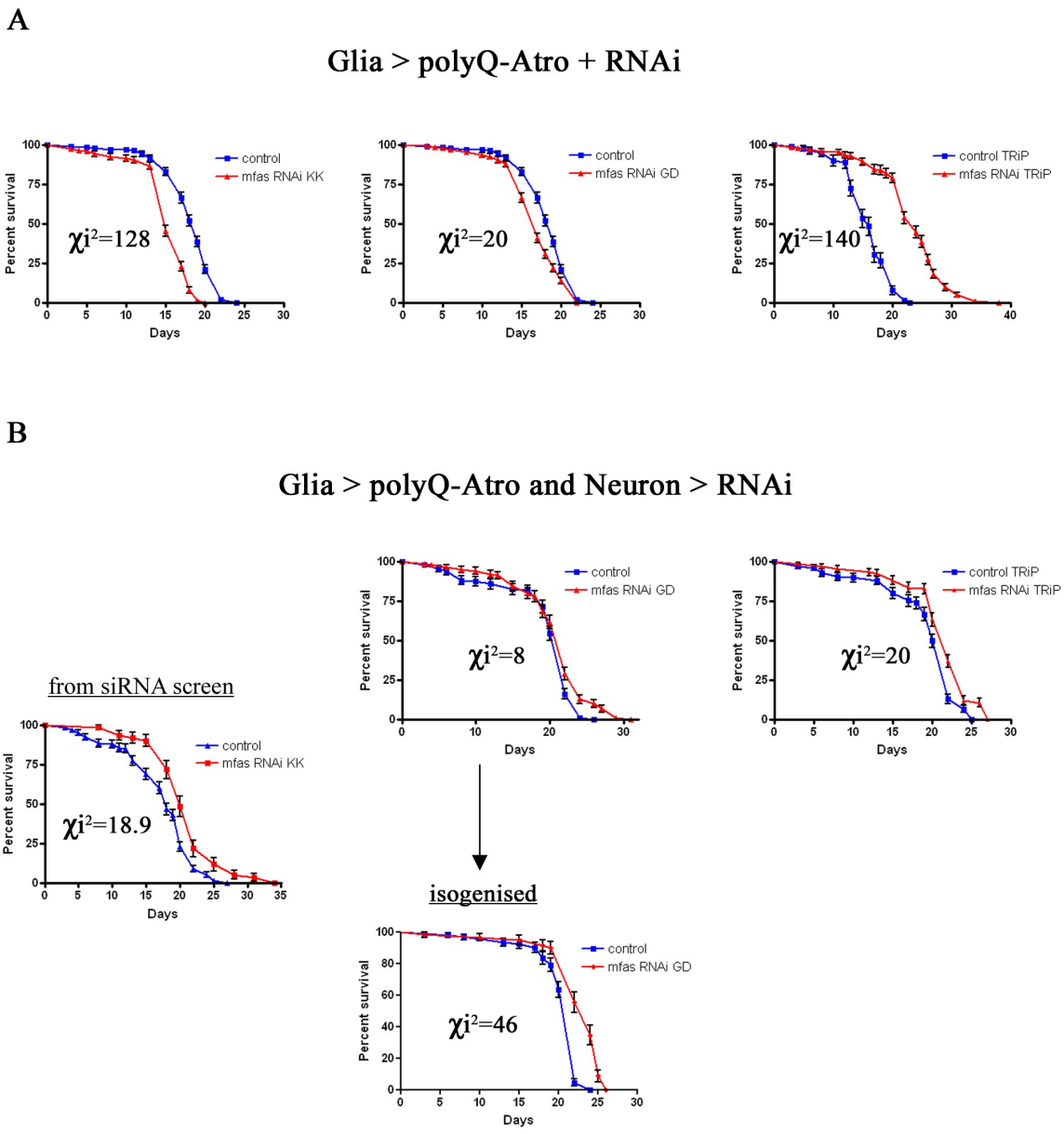


Figure 4.4: *mfas* effects in glia and neurons. (A) Lifespans of the different mfas RNAi lines expressed in adult glial cells beside the *polyQ-Atro*. The genotypes are: *Repo-Gal4,TubGal80^{ts};UAS-sAtro-pQ* crossed either to *w^{1118}* or the RNAi of the named gene. **(B)** Lifespans of the same RNAi lines but expressed in adult neurons while *polyQ-Atro* remains expressed in adult glia. The genotypes are: *Elav-Gal4,Repo-LexA;UbiGal80^{ts},LOT-sAtro-pQ* crossed to either *w^{1118}* or the different RNAi lines of mfas. Only females were used for the neuronal expression of the RNAi since the drivers are on the first chromosome. The non-isogenised lifespans were done with 120 to 200 flies and the isogenised and siRNA screen ones with 60 to 90 females. The statistical analysis were done as previously described.

4.2.3.2 *caps* and *trn* genes interact with glial *polyQ-Atro* expression

Another candidate I followed up from the RNAi screen is *capricious* (*caps*). *caps* was in the 6th position of the suppressor genes, with a final Chi square value of 45.96. *caps* has been extensively studied in development along with the close related gene *tartan* (*trn*), which has been shown to be complementary and shares 65% of its extracellular domain with *caps* consisting in 14 LRR repeats for *caps* and 10 for *trn*. They have been involved in dendrite targeting in the olfactory bulb (Hong et al. 2009) and synapse formation and target recognition at the NMJ (Shishido 1998, Kurusu et al. 2008, Kohsaka et al. 2009). On the basis of these complementarities in their functions, several *trn* RNAi lines were analysed along with 2 new RNAi lines against *caps*. The RNAi lines were first assessed in adult neurons in the same set up than for the siRNA screen where *caps* was identified. Similarly to the line assessed in the RNAi screen (3046/GD), the new GD line against *caps*, numbered 27097/GD, also increases the lifespan though to a lower extent compared to control, with a Chi square value of 16.5 and a smaller difference of median survival (**Figure 4.5A**). However, the TRiP line did not give any phenotype compared to the *mCherry* RNAi line used as control for this genetic background. *caps* possesses several isoforms, however, all the RNAi lines target exons that are present in all isoforms, ruling out a potential specificity of isoforms differentially downregulated. Isogenisation of the GD line 27097 did not change the effect and the difference of median survival remained similar to prior isogenisation (data not shown). It is thus possible that the effect of this GD line is weaker than the previous one used for the screen regardless of the isogenisation of the genetic background.

Then, *trn* having a complementary role, two RNAi lines have been assessed in the same set up. Those two RNAi lines are from the Bloomington collection, numbered 28525 and 50520. None of them had a phenotype when expressed in neurons beside glial *polyQ Atro* expression, with respective Chi square values of 0.1 and 1.5, again with 130 flies assessed for each genotype (**Figure 4.5A**). These results suggest that *trn* is unlikely to have a role in neurons in the impaired interactions between diseased glia and neurons.

trn is known to be expressed in different cells than *caps*, leading to specific target recognition. I then wondered whether *trn* could be expressed in glial cells and would potentially interact with caps in a non-cell autonomous mechanism. Thus, all the RNAi lines against *trn* and *caps* were expressed in glial cells in combination with *polyQ Atro*. Both GD lines of *caps* did not have any phenotype, with their Chi square values of 5 and 0.1 respectively and the lifespan curves almost completely overlapping (**Figure 4.5B**). The isogenisation process of these two lines did not change the lifespan and the curves were still overlapping (data not shown). However, the TRiP line increases the lifespan, with a Chi square value of 33. For the RNAi lines against *trn*, both rescue the phenotype, with Chi square values of 65 and 110 (**Figure 4.5B**). All the lifespan were done using 160 flies for the RNAi lines but only 80 for the TRiP control. This set of experiments would need to be repeated to be confirmed in order to validate the lifespan of the control with a higher number of flies. However, in the set up where the RNAi lines are expressed in neurons, the RNAi TRiP lifespans were overlapping with the TRiP control. It is thus unlikely that the RNAi against mCherry is toxic for the cells. However, it is possible that it has off target effects specifically in glial cells even though they are supposed to be designed to avoid them. Another control line in the TRiP background would be needed to confirm a biological effect of the TRiP RNAi lines when expressed in glial cells.

In the presence of a number of contrasting effects of the different RNAi lines it is difficult to conclude whether *caps* and *trn* play a role in glia-neuron communication during neurodegeneration. Downregulation of *trn* in glial cells appeared to have a strong and reproducible phenotype, however this is based on two TRiP lines and in this paradigm, all TRiP lines for *caps*, *trn* and *mfas* displayed the same effect, which raises some doubts about the appropriateness of the control lifespan. Further experiments need to be done using different RNAi lines, especially against *trn*. It would be ideal to confirm the rescue in glia polyQ condition using lines from the VDRC collection, which were not available at that time. The knock-down efficiency for each RNAi would also need to be assessed to get a clearer picture.

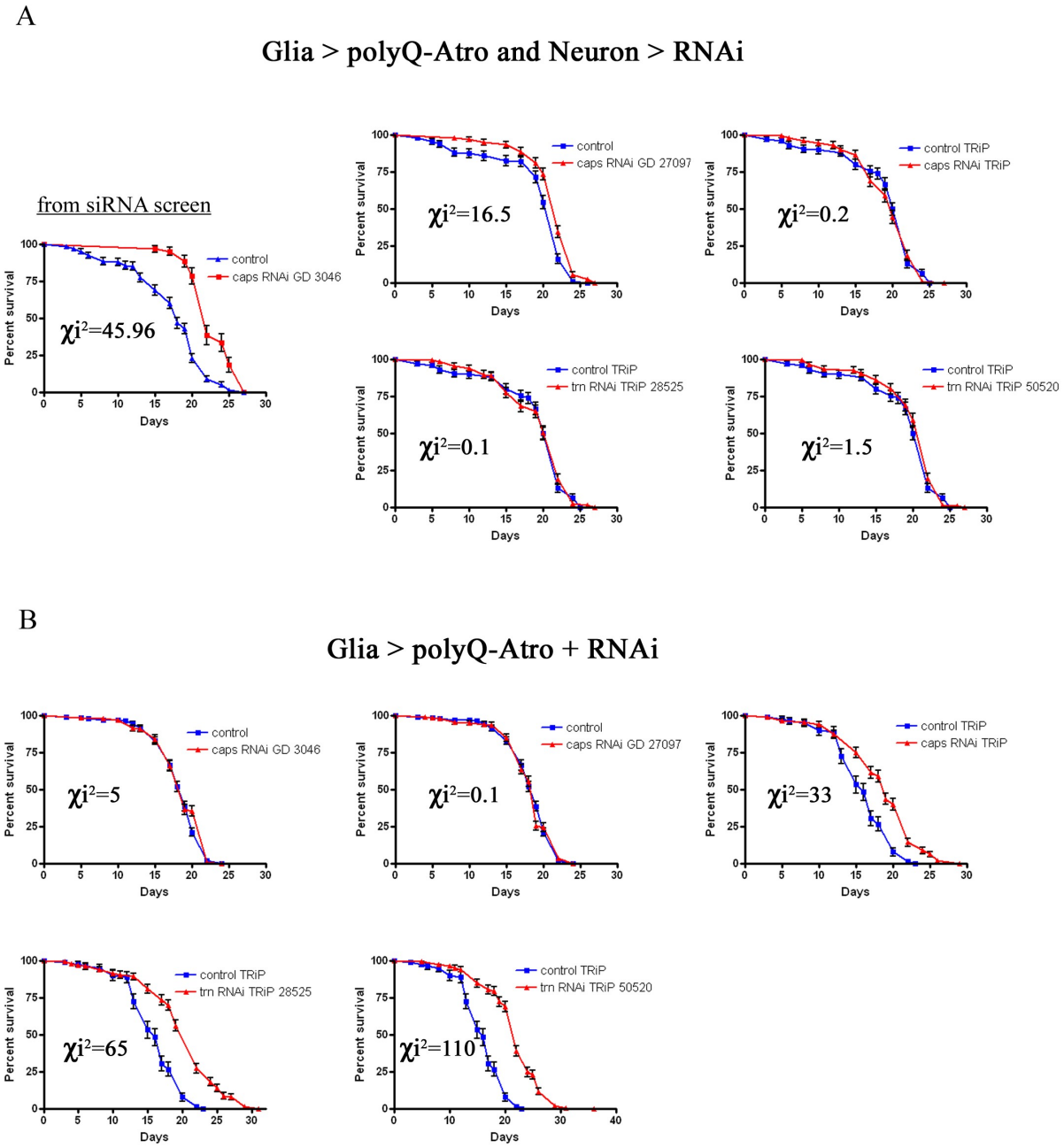


Figure 4.5: *caps* and *trn* effects in glia and neurons. (A) Lifespans of *caps* and *trn* RNAi lines expressed in adult neurons beside *polyQ Atro* expressed in adult glia. The genotypes are: *Elav-Gal4, Repo-LexA;UbiGal80^{ts},LOT-sAtro-pQ* crossed to either *w^{1118}* or *trn/caps* RNAi lines. Only females are assessed because of the presence of the drivers on the X chromosome. **(B)** Lifespans of the same lines but expressed in adult glia beside *polyQ Atro.* The genotypes are: *Repo-Gal4,TubGal80^{ts};UAS-sAtro-pQ* crossed with either *w^{1118}* or *trn/caps* RNAi lines. All lifespans were done with between 120 to 200 flies appart from the one from the siRNA screen that was done with 60 female flies. The statistical analysis were done as previously described.

4.3 Discussion

The use of an unbiased screen for new genetic interactions allows the discovery of many different pathways that could be involved in cell-cell communication. In this set up, a toxic protein is expressed in one cell type, glial cells, and RNAi are independently expressed in another one, neurons. Thus, it is rather challenging to uncover genes that could modulate the phenotype in a non-cell autonomous mechanism, in addition to the variability to RNAi efficiency. The fact that out of a thousand downregulated genes in neurons only 12 decreased even further the lifespan, it shows that likely the organism is able to cope efficiently with defective pathways, possibly by redundancy mechanisms or a high number of ineffective RNAi lines. It has to be mentioned that the expression of glial *polyQ Atro* has a strong phenotype, reducing the lifespan by nearly 50%, which is dramatic. And depending on the strength of the RNAi, the turnover of the mRNA and the half-life of the protein, it can take days before having a knock-down strong enough and then could take more days to see an effect in behaviour. All of these could explain why the modulation of such a strong phenotype can be challenging. However, 29 RNAi lines were able to modulate it, with 17 rescuing the lifespan to up to 25%. The candidate genes obtained following the RNAi screen were not enriched in a specific type of function. It suggests that many pathways in neurons are affected by the glial toxicity, which is in line with the large transcriptional alterations triggered by *polyQ Atro* expression (Napoletano et al. 2011). Those two cell types communicate at different levels, either directly by cell-cell contact via membrane proteins, by secretion of vesicles or by proteins that will bind to receptors. Also, it could be considered as indirect communication when glial cells are not doing properly some of their functions such as neurotransmitter clearance/recycling, which will certainly affect non-autonomously neuronal functions. The examples of the two strongest suppressors and the chosen candidate genes show the range of pathways in neurons possibly affected by impaired glia.

The three candidate genes were selected based on their related function in cell adhesion/communication, which is of high interest in our understanding of glia-neuron

interactions in a disease context. Furthermore, *mfas* downregulation was predicted from the miRNA screens to have opposite effect in glia and in neurons, also confirming the RNAi screen result. In the case of *caps*, the preliminary result on *trn* downregulation effect in glia led us to investigate potential direct interaction between both membrane proteins. The other top candidates were involved in complex interactions such as *VhaSFD*, which is involved in vesicular acidification, thus, could be responsible of many different impairment of signalling pathways at the same time (Thomas et al. 2006, Kolotuev et al. 2009, Vaccari et al. 2010, Dupont et al. 2012). Cell-cell direct interactions through membrane proteins seemed to be natural candidates to understand communication between glia and neurons, which would be provided by the investigation of genes such as *mfas*, *caps* and *trn*.

Except for the result of the TRiP line, *mfas* has shown opposite effect on the lifespan whether it is downregulated in glia or in neurons with the RNAi lines from the VDRC collection. The analysis of the miRNA screens have revealed that several miRNAs have this differential phenotypes, being enhancer in one cell and suppressor in another cell, which is really interesting. The targets of these specific miRNAs are currently being analysed to see if such genes can reproduce this specific effect. According to the preliminary data obtained with *mfas*, a predicted target of *miR-8* (being one of those miRNAs having cell-specific phenotypes), it is possible that this Fasciclin-I related gene could be such a candidate. However, it is also possible that those miRNAs have their effects by targeting different genes in both cell types since their gene expression profiles are very different. Thus, a target gene would be responsible or at least participate in the decrease of the lifespan in glia but another one, not expressed in glia would do the opposite in neurons. *mfas* would need to be further investigated as one RNAi line has an opposite effect from the two others in glial cells. The genetic background of this RNAi-TRiP line is different, being on (*y, v)* background, and will have to be analysed more thoroughly. However, based on the VDRC RNAi lines lifespans, it is possible that *mfas* has a different functions in glia and neurons that lead to opposite effects. *mfas* seems necessary in glia and could participate to the establishment and maintenance of glial shapes. Indeed, Fasciclin-related genes have been involved in neuronal arborisation by homophilic adhesion (Schuster et al. 1996). It is thus possible that disrupting glial shapes is harmful for the CNS

function while increasing it in neurons could lead to better synaptic plasticity that could delay neuronal impairment. We know now from the I. Salecker group that *mfas* is expressed at least in the glial cells of the optic lobe in pupal stage and could have a role in the arborisation of the epithelial glia. It is thus possible that *mfas* help to fasciculate the axons during metamorphosis but it remains to be proven whether it has a role in the adult, possibly by maintaining a correct fasciculation of some tracts.

In lifespan especially, genetic background is an important factor and usually the lines have to be backcrossed to an isogenised stock in order to limit its impact on the phenotype. In the scale of a screen where a thousand genes are assessed, it is not feasible to isogenise such an amount of lines. However, 90% of the lines did not show any phenotype in the first screen showing that the big majority of the lines, even without being backcrossed did not show excessive variability. However, for mild effect such as *mfas* or *caps*, it is necessary to isogenise stocks in order to be confident on the biological significance of the phenotype observed. In the case of *mfas*, it has revealed a bigger effect, whereas this did not achieve any difference for *caps*. Moreover, increasing the number of flies to few hundreds on isogenic background would help to differentiate genetic variability from biological effects of an RNAi, especially when the defects are subtle. A more complex issue is that of the different effects of RNAi lines coming from different collections and their relative efficiency and potential off targets. A careful analysis of the extent of protein downregulation and of the effect of genomic mutations would be needed to better understand this intricate network of effects but that would require antibodies against the proteins, which are currently unavailable. However, should the phenotypes of *caps* and *trn* downregulation be confirmed, it would be very interesting to investigate the role of a possible interaction between these two membrane proteins in the communication between glia and neurons in health and disease. So far, they have been mostly studied in neuron-muscle interactions at the NMJ or target recognition in the olfactory bulb during development. It is still possible that at least *trn* is also expressed in some glial cells and tightly interacts with some neurons. More work would need to be done to further characterize these potential glia-neuron interactions that seem to be of importance in a disease context.

Chapter V:

repo expression in adult glia is necessary for CNS homeostasis

5.1 Introduction

5.1.1 Using miRNAs as a tool to discover interacting genes

siRNAs are supposed to be specific to a single gene and have few off-target effects predicted. However, this has the consequence that a thousand lines need to be assessed to screen a thousand genes, making big screens a hard and long work that requires months of experimental work. Another way to downregulate genes is using microRNAs. microRNAs are endogenously expressed in eukaryotic cells and in some viruses and are known to be able to downregulate several genes through smaller RNA sequence alignment to the mRNA (7 nucleotide) than siRNA. Also, the silencing effect does not necessarily require perfect complementation between the miRNA and its target mRNA. Some miRNA are predicted to have hundreds of potential targets. Thus, another strategy designed in the lab was the overexpression of miRNAs instead of siRNAs. By experimental and bioinformatic predictions, it would be then possible to narrow down potential targets of positive miRNA hits in order to increase the ratio of genes assessed per positive candidates, which was very low in the RNAi screen. Actually, a very recent study that has been published has used the same strategy and has discovered genes involved in odorant receptor expression (Bhat et al. 2016).

The *Drosophila* genome possesses 256 miRNAs including 34 miRtrons (Kozomara et al. 2011, Kozomara et al. 2014). However, not all of them have been confirmed to be expressed. Several collections have been developed for *UAS-miRNA* or knock-outs. One of them includes around 140 *UAS-miRNAs* and has been kindly shared with our lab by Eric Lai (Fulga et al. 2015). Theoretically, screening 140 miRNA overexpression lines would give access to thousands potential downregulated genes. This strategy has been used to elaborate different screens in the lab. A similar screen to the RNAi one has been done by other lab members, with this time its control without *polyQ Atro* expression. Moreover, two other ones have been done, using a cell-autonomous paradigm. The *UAS-miRNA* lines are co-overexpressed in glia beside *polyQ Atro* or again as control without *polyQ Atro*

expression. The miRNAs having an effect in both conditions (with or without *polyQ Atro*) would be considered non-specific to *polyQ Atro* expression. Once the miRNAs were classified according to their effect on the lifespan and in which screens they had a phenotype, their targets were further analysed (**Figure 5.1**).

The miRNA screens and their analysis have been carried out by other members of the lab. However, as an example, *mfas* came out of it as being a target of *miR-8*, which has an opposite effect on lifespan whether it is overexpressed in glia or in neurons in our DRPLA model, thus leading to the investigation of *mfas* in the previous chapter.

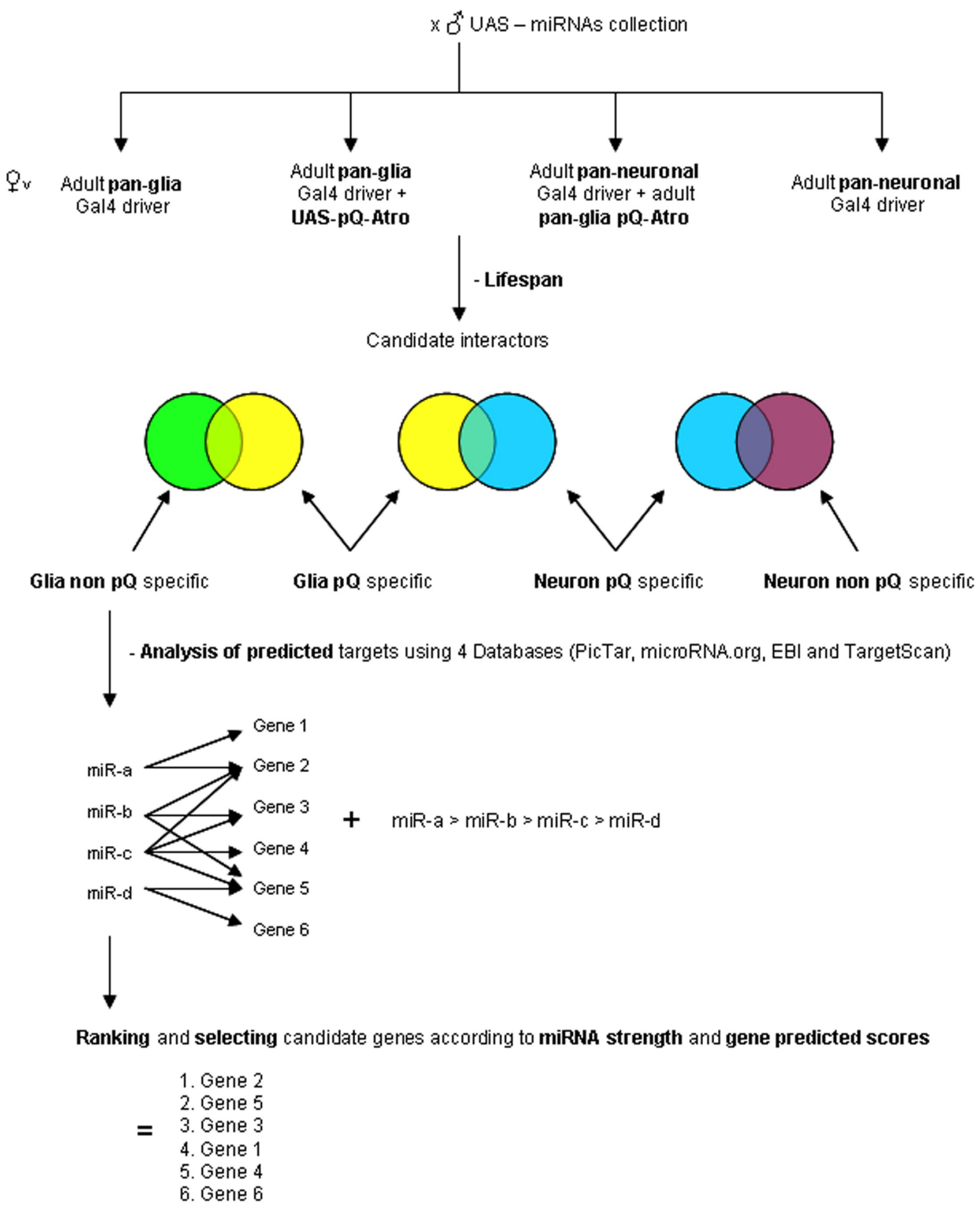


Figure 19: Strategy, design and analysis of miRNA screens. Lifespan is done using the progeny out of the designed crosses indicated at the top of this schematic. Lifespans are done as previously described. Two independent lines are used for each miRNA and the average Chi square for each miRNA is used to assess the strength of the phenotype. The scores for each gene from each miRNA is determined by each website. The ranking is established by combining the strength of each miRNA phenotype targeting a certain gene, the number of miRNA targeting this gene and the predicted score from each database for each of those miRNA.

5.1.2 *miR-1* is expressed in the mesoderm during embryogenesis

Beside the RNAi/miRNA screens carried out in glia and neurons in disease condition, I also focused on potential miRNAs that would be interesting and relevant to the general function of glial cells in *Drosophila*. Understanding general glial cell functions will help deciphering mechanisms and pathways that can be disrupted in neurological disorders. One of those miRNAs is *miR-1*, obtained from preliminary data by other lab members. When overexpressed in adult glial cells, the lifespan was dramatically reduced. This effect was obtained using two different *UAS-miR-1* lines, reassuring on eventual artefacts due to the insertion of the transgene. Using the different miRNA databases available (PicTar, microRNA.org, EBI/Microcosm Targets and Target Scan), the list of potential targets of *miR-1* was analysed critically, with a special focus on genes that may be relevant in the glial cells, where *miR-1* was overexpressed. One of those target genes was *repo*, which is the only specific pan-glial marker constitutively expressed throughout the entire life of the *Drosophila* and the interaction between *miR-1* and *repo* will be further investigated in this chapter.

The expression of *miR-1* has been mostly characterized in the *Drosophila* embryo. From stage 5 to 10, it is specifically expressed in the mesoderm. At stage 12 and 13, it is visible in all differentiating muscles, including somatic muscles and pharyngeal muscles (Aboobaker et al. 2005). It has been shown that *miR-1* is directly activated by the promesodermal transcription factor *twist* during early embryogenesis and later maintained in myogenic cells by the promyogenic transcription factor *Mef2* (Sokol 2005). Furthermore, the *miR-1* expression in cardiac and skeletal muscle precursor cells is conserved across species (Lee et al. 2001), including mammals (Sempere et al. 2004). In a microarray analysis, Lim et al. show that some miRNAs, including *miR-1*, could downregulate a large number of target mRNAs especially enriched in genes normally expressed in different tissues, non-muscle cell genes in the case of *miR-1* (Lim et al. 2005). This analysis shows that miRNAs could help in maintaining specific cell type identity during development by repressing specific genes that could potentially change the fate or function of a cell. Laneve et al. strengthen this hypothesis by showing that *miR-1* could also be activated by *gcm*

during early embryogenesis (Laneve et al. 2013). At this developmental stage, *gcm* is important in blood cells development, before being the key glial-fate determinant expressed at later stages in the neuroectoderm. These discoveries give some ground to speculating about *miR-1* function in the regulation of blood-muscle-glial cells differentiation. It would allow the hemocytes to remain hemocytes early in development and muscle cells to remain muscle cells during late development by inhibiting genes that could change fate or deregulate cell function. Indeed, *gcm* is also known to be the first glial cell-fate determinant and directly triggers the transcription of *repo*, responsible for the terminal differentiation of glial cells.

5.1.3 *repo* expression is tightly regulated during embryogenesis but its function is unknown in adult *Drosophila*

As mentioned in Chapter I, *repo* has been extensively studied during embryogenesis. Its expression level is tightly controlled both at the transcriptional and post-transcriptional level by *gcm* and *dCBP* until *repo* takes over the control of its own expression by a feedforward-loop (Flici et al. 2014). It has also been shown that *repo* triggers the expression of secreted *wg* by glial cells at the NMJ in larval stage (Kerr et al. 2014), regulating glutamate receptor clustering and synaptic physiology.

However, beside its role during development, very little is known about its function in adult *Drosophila*. The only study investigating a potential role of *repo* specifically in the adult has demonstrated that it is important for long-term memory via glial transcription (Matsuno et al. 2015). *repo* is a terminal differentiation factor, triggering specific glial programs during late embryogenesis. It remains expressed, constantly, at high levels in all glial cells in the adult *Drosophila*. The *Gal4* line used for pan-glial transgene expression is actually a knock-in in the endogenous *repo* locus. This transgenic line is broadly used for all *Drosophila* stages, including adult. The question is why is it still necessary in the adult?

This chapter aims at understanding two main mechanisms. The first one is deciphering the predicted relationship between *miR-1* and *repo* during development. The complex expression pattern of *gcm* in different cell types has been extensively analysed. *miR-1* could potentially be a missing key in the understanding on how *gcm* is expressed in mesodermal cells without triggering ectopic *repo* expression. This hypothesis is in line with the microarray analysis done in the study of Lim et al. (Lim et al. 2005) about miRNAs downregulating genes specific to other tissues, such as a glial cell fate determinant being wrongly expressed in the mesoderm. On the basis of this potential link between *miR-1* and *repo* determined using *miR-1 KO -/-* embryos and luciferase assay, and the effect of glial *miR-1* expression on adult lifespan, the second main question that arose is: what is the function of *repo* in adult glial cells given its maintained expression? Does it still have a role in cell fate determination or maintenance allowing a certain flexibility of cell identity in the adult CNS? And/Or does it still regulate specific genes' expression, as it is the case during development, for terminal cell differentiation?

5.2 Results

5.2.1 *miR-1* downregulates *repo* and participates in its restricted expression pattern during embryogenesis

5.2.1.1 Ectopic expression of *miR-1* in glial cells has a dramatic effect on *Drosophila* at larval and adult stages and targets *repo* both *in vitro* and *in vivo*

Preliminary data obtained in the glial miRNA screen showed that the adult specific overexpression of *miR-1* in glial cells using *Repo-Gal4* driver recombined with *UbiGal80^{ts}* was reducing the lifespan. I first confirmed this result increasing the numbers of flies assessed for the lifespan, using ~ 250 for both control and *miR-1* overexpression instead of 60 in the screen in order to increase confidence in the results. As seen in **Figure 5.2A**, the adult lifespan is reduced by 50%, from a median survival of 25 days in control to 13 days in *miR-1* overexpression condition ($p<0.0001$).

Since adult glial *miR-1* expression has a dramatic effect on lifespan, I also assessed the effect of its expression in glial cells during development. Using temporal control of transgene expression, *miR-1* was expressed specifically from first instar larvae (L1) onwards. The Tubby marker (oval shape instead of elongated larvae) was used to discriminate wild-type from *miR-1* larvae. All *miR-1* larvae died at third instar larvae (L3), when the larvae come out of the food to enter pupation for metamorphosis. *miR-1* L3 larvae bodies were thinner than non tubby wild type L3 larvae (data not shown). The brains of the larvae overexpressing *miR-1* in all glia were then dissected prior to death and stained for *repo*, the pan-glial marker. The brains seemed obviously smaller and the VNC thinner and more elongated (VNC data not shown). The staining of *repo* confirmed this observation by comparing the size of the brain lobes using confocal imaging (**Figure 5.2B,** white circles in Z-projection panels). Also, the glia forming the presumptive lamina layer (outer and inner chiasm glia) failed to form a proper bi-layer pattern as compared to wild-type, interestingly very similar to the defects observed in *repo* mutant larvae (Xiong et al. 1994). (**Figure**

5.2B, arrows in confocal section panels). This phenotype strengthens the idea that *miR-1* affects important glial function both during development and adult stages.

According to several databases, *repo* was predicted to be a target of *miR-1*. This prediction was consistent with western blot using adult fly heads expressing *miR-1* specifically in glial cells (**Figure 5.2C**). *repo* is indeed significantly downregulated by 85% when *miR-1* is overexpressed in glia, with a P-value of 0.011. The direct effect of *miR-1* on *repo* mRNA 3'UTR was assessed *in vitro* by Luciferase assay. *repo* mRNA possesses two known *miR-1* binding sites in its 3'UTR. Thus, three different plasmids have been designed by our collaborators at IGBMC in Strasbourg. They all express the coding sequence of the Luciferase. However, they differ in their 3'UTR sequence. One possesses the wild-type 3'UTR of *repo* to confirm that *miR-1* can downregulate the Luciferase activity. One control plasmid with the Luciferase 3'UTR in order to assess that *miR-1* does not decrease independently the Luciferase activity. And a third one with the *miR-1* sites of the *repo* 3'UTR mutated to see whether *miR-1* has its activity through these specific sites and point toward a direct inhibition. The *miR-1* expression was controlled by a *tubulin* promoter and thus was expressed post transfection. The expression of the other plasmids was controlled by copper sulphate activation of the *pMT-Gal4* plasmid. This allows the expression of *miR-1* before the expression of the Luciferase plasmid in order to build up the accumulation of *miR-1* and increase the efficiency of the knock-down. The Luciferase assay shows that *miR-1* does not downregulate the Luciferase activity in the absence of *repo* 3'UTR, validating the assay. However, the Luciferase activity from the mRNA with the *repo* 3'UTR is significantly reduced whereas with the mutated *miR-1* sites it is rescued (**Figure 5.2D**). Compare to the western blot, where the Repo protein level is strongly decreased, the signal is only decreased by half. In the luciferase assay, the time frame is a lot shorter and it is thus possible that the maximum downregulation has not been reached. In any case, these results point strongly toward a direct downregulation of *repo* by *miR-1* through its identified 3'UTR sites as predicted by the identification of *miR-1* sites in *repo* 3'UTR using different websites.

To confirm this result *in vivo* a *UAS-Repo* line with the 3'UTR from SV40 was used in order to express the Repo protein without influence from *miR-1*. The sensitivity of this line for *miR-1*, called *Repo#9* ((Matsuno et al. 2015), referred to as *repo-myc* in the mentioned paper) was first assessed by western blot. While *miR-1* downregulates strongly the endogenous *repo*, it does not affect the expression of *Repo#9* transgene, controlled by the myc tagged Repo level (**Figure 5.2E**, lower panel) and the level of expression is similar to that of wild-type (**Figure 5.2E,** upper panel). As already observed in the previous study, using this myc-tag *Repo#9* line, a doublet can be seen by western blot. This could come from potential post translational modifications such as phosphorylation. However, since this line has been proven to rescue *repo* mutants, the formation of this doublet does not seem to affect Repo-myc function (Matsuno et al. 2015).

The next question was then whether the *miR-1* phenotypes were caused by the downregulation of *repo*. In order to determine whether the effects of *miR-1* on lifespan and on larval brain development were due to *repo* downregulation, these experiments were repeated with the co-expression of *Repo#9* beside *miR-1*. In the larval stage experiment, the overexpression of this *miR-1*-independent *Repo#9* line did not rescue the L3 lethality or brain size (100% died at L3 larval stage, data not shown). However, in adult, even though the median survival is 13 days for *miR-1* and 11 days for *miR-1 + Repo#9* conditions, a tail in the *miR-1 + Repo#9* genotype lifespan shows a rescue for 20% of the population. When such a tail is present, the maximum lifespan can be calculated using the median survival of the last 10 to 20% of the population (Slack et al. 2011). By taking the last 20% of the population (respectively 74 and 66 flies for *miR-1* and *miR-1 + Repo#9*) *Repo#9* expression rescues the lifespan by 33%, going from a median survival of 15 days for *miR-1* to 20 days for *miR-1 + Repo#9* ($p<0.0001$). This type of lifespan with such a tail could indicate an age-related effect on the *Drosophila* organism. It is also possible that there is a delicate balance between the survival and the death of the organism in this condition and only a low percentage of the population survives to it. This may suggest a minor rescue in the individuals that for additional reasons are more resistant to the expression of *miR-1.* In order to decipher further the implication of *repo* in the mechanism leading to the fly death via glial *miR-1*, a *UAS-Repo* line lacking not only the *repo* 3'UTR but also a large part of

the transcriptional activation domain, called *Repo#14*, was used ((Matsuno et al. 2015) referred to as *repo-mycΔAD302*, potentially acting as dominant negative). This line provides a sensitized background for *repo*, challenging glial cells on the *repo* "pathway" beside the *miR-1* effect. The lifespan of flies expressing *Repo#14* only have a mild, although significant, decrease compared to control, showing a mild toxic effect of this line (**Figure 5.2A**). However, when expressed beside *miR-1*, a much greater reduction of the lifespan is observed with a chi square of 371, compared to *repo#14* alone compared to control with a chi square of 33 (note that the Chi square values are very high since the number of flies assessed were greater than 240 per genotype). This result suggests that the function of *repo* as transcriptional activator is important when *miR-1* is overexpressed in glia, in order to maintain a certain viability of the organism.

Both results of the attempted rescue in adult and developing glia, suggest that other factors, in addition to *repo*, are responsible for most of the effects provided by the ectopic expression of *miR-1* in glial cells. It is well established that miRNAs can downregulate many genes at the same time, thus it is possible that another gene, or even more likely a combination of several genes, are responsible of the different phenotypes. It is also possible that the downregulated genes involved in each phenotype, larval and adult stage ones, are different.

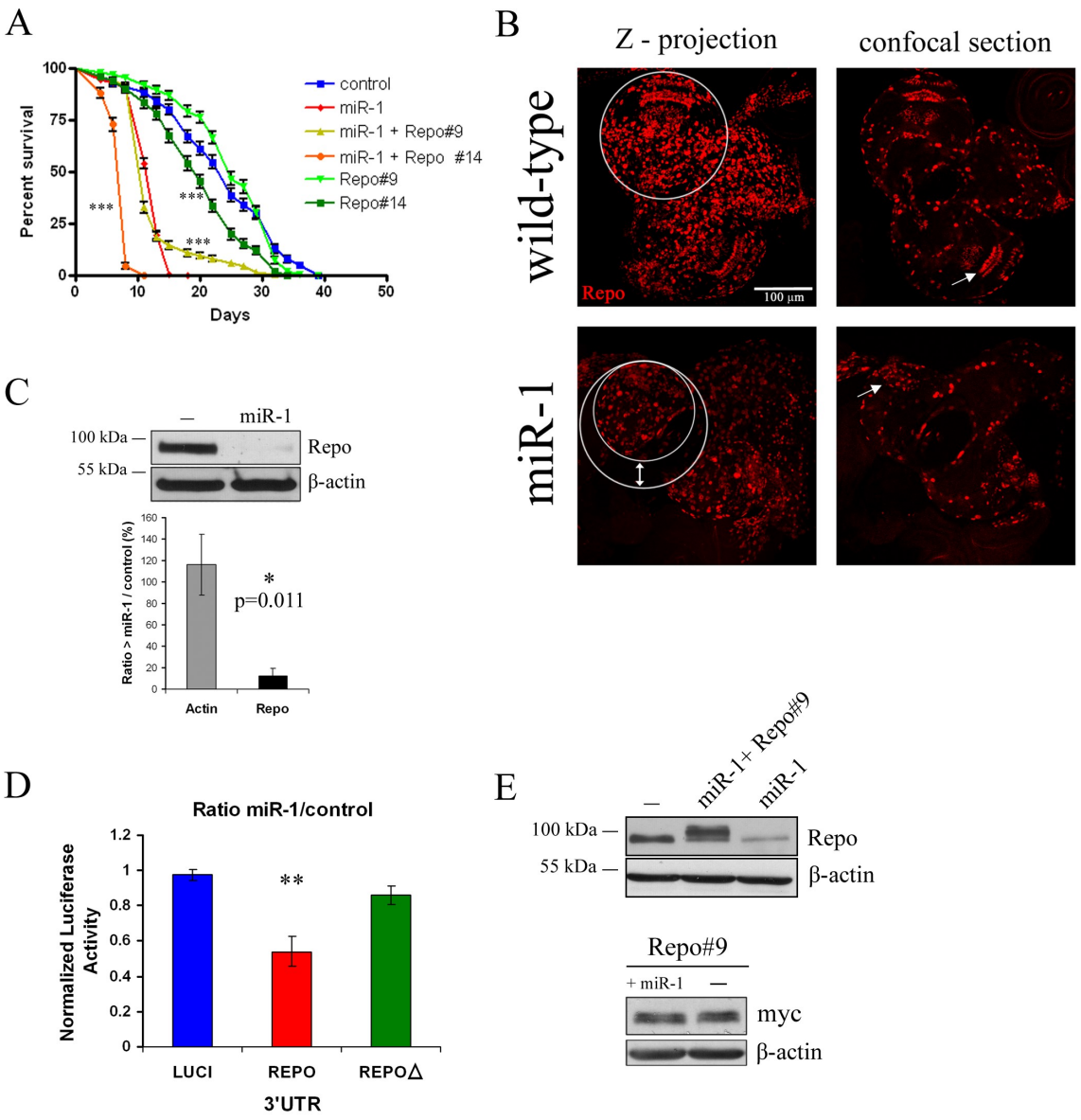


Figure 5.2: *miR-1* expression in glial cells downregulates *Repo* in vitro and in vivo and decreases larval L3 brain size. (A) Lifespan of flies expressing *miR-1* and *miR-1* sites mutated *Repo#9* or a truncated version *Repo#14*, in adult glia. The genotypes are: *Repo-Gal4;UbiGal80^{ts}* crossed to *UAS*-(legend). *w^{1118}* is used as control. More than 180 flies were assessed per condition. Statistical analysis was done using Prism GraphPad. ***: p<0.0001, *miR-1+Repo#14 is* compared to *miR-1* and *Repo#14 is* compared to control. The significance of *miR-1 + Repo#9* against *miR-1* applies for the 20% last percent of the population. **(B)** Confocal pictures of L3 larval brains from control larvae or expressing *miR-1* in glia from L1 to L3 (similar genotypes as **(A)**. Arrows in confocal section panels show defects in chiasm glia pattern in *miR-1* compared to wt. **(C)** Western blot and quantification of *Repo KD* by *miR-1* expression using the pan-glial driver *Repo-Gal4* using *Gal80^{ts}* to allow specific adult expression during 10 days. 3 independent biological replicates have been quantified, loading the equivalent of 5 brains in each condition. The statistical analysis has been done using t-test. **(D)** Luciferase assay using S2 cells transfected with luciferase coding sequence with different 3'UTRs, cotransfected with or without miR-1 plasmid. The assay has been done in triplicates. The statistical analysis has been done using one way ANOVA. **: p<0.01. **(E)** Top panel: Western blot of *Repo#9* expression in the presence of *miR-1* after 10 days at 29°C, using anti-Repo Ab. Bottom panel: Western blot controlling *Repo#9* levels using anti-myc Ab in the presence or not of *miR-1*. The same genotypes as in **(A)** were used. Error bars in graphs represent standard error.

5.2.1.2 *miR-1* is involved in the regulation of glia-specific *repo* expression during embryogenesis

So far, the relationship between *miR-1* and *repo* was in the context of an ectopic expression of *miR-1* in glial cells using the pan-glial driver *Repo-Gal4*, where it is not supposed to be expressed, at least during embryogenesis where its expression pattern has been characterized. *miR-1* is normally expressed in the mesoderm, which will later on generate hemocytes (future "blood" cells) and muscle cells. The question is why *miR-1* would be able to downregulate *repo*, a gene not supposed to be expressed at the protein level in the mesoderm. Is it the result of an artefact of overexpression, which allows the miRNA to bind less specifically to some mRNAs, which in physiological conditions would not happen? Or is it an interaction that happens during development in order to maintain mesodermal fate in some cells susceptible to express *repo* by blocking the RNA translation?

As mentioned in **5.1.1**, *miR-1* expression in early embryogenesis, when hemocytes are being formed, is regulated by both *twist* and *gcm*. However, *gcm* is also able to directly trigger *repo* transcription in the neuroectoderm. There are many ways to repress transcriptional activation, by the binding of transcriptional repressors for instance. But it is also possible to repress the protein expression at the post transcriptional level and the discovery of the role of some miRNAs on the control of cell fate determination goes in this direction (Tsuyama et al. 2015).

It has been shown by our collaborators at IGBMC, that in a *twist* mutant (twi^1 -/-, (Castanon et al. 2001)) background embryo, there is an overlap between *repo* and *srp* in around 10 cells per embryo in the mesoderm (Trebuchet et al, 2016, manuscript in preparation). *srp* is used as an early marker for hemocytes. To answer the above mentioned question, a *miR-1 KO* embryo line was used to see whether there would be such an overlap between these two different markers in the mesoderm. The co-staining with Repo and Srp antibodies of *miR-1 KO* embryos revealed a very similar phenotype to the *twist* mutant embryos (**Figure 5.3A**). In the heterozygous *miR-1* embryos (-/+), there is an increase in

cells positive for both markers but it did not reach significance. However, in homozygous (-/-) embryos, 8 to 12 cells on average were positive for Repo and Srp staining per embryo in the mesoderm, phenocopying the *twist KO* phenotype (**Figure 5.3B**). This result suggests that *miR-1* is important for proper separation of the hemocyte and glial lineage and this might happen via downregulation of *repo* in physiological condition. It is likely that this interaction occurs in the early stages of embryogenesis, when *gcm* is transiently expressed in the mesoderm. The phenotypes of both *twist KO* and *miR-1 KO* are weak. This may suggest that there are other potential redundant repressors acting to prevent ectopic *repo* expression in the mesoderm.

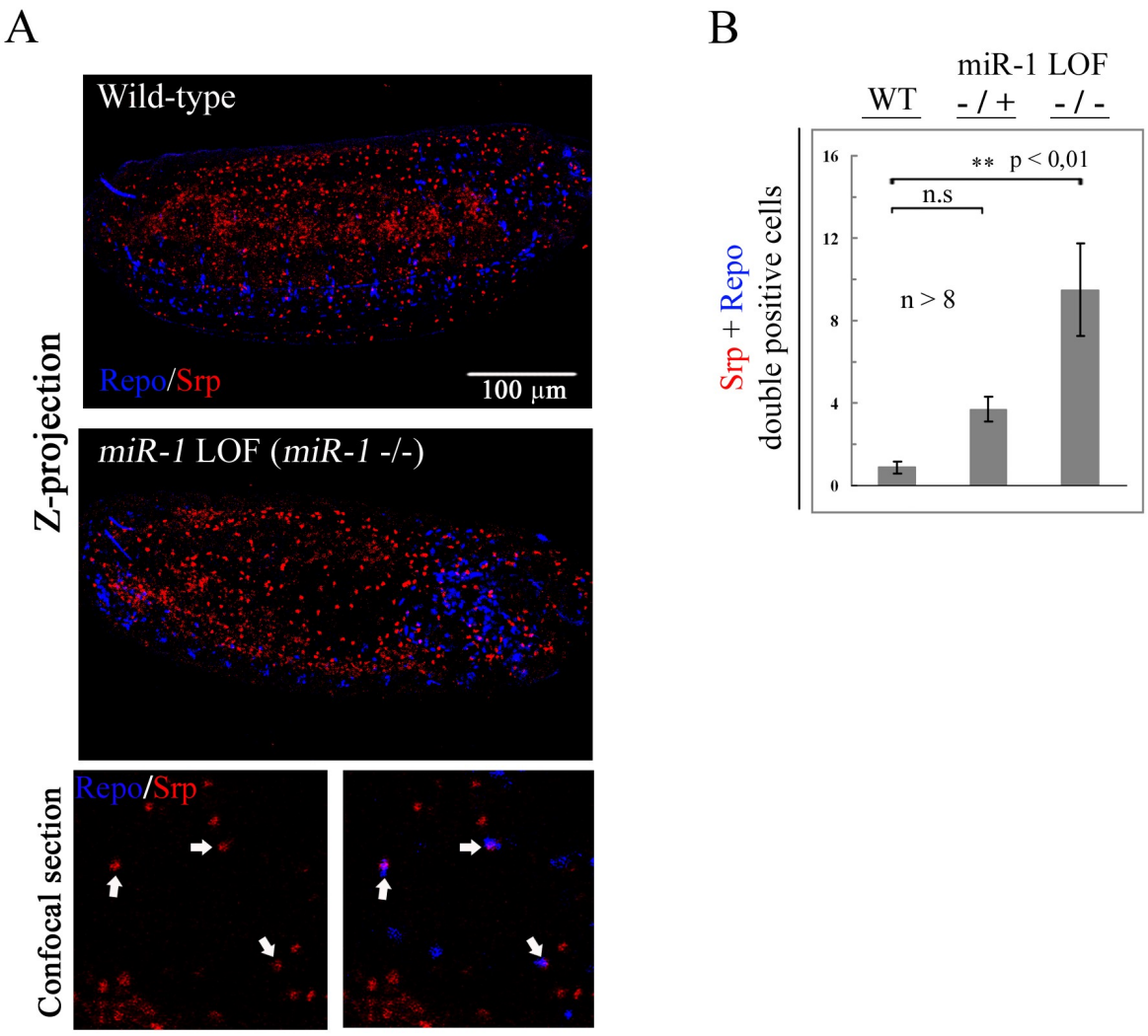


Figure 21: *miR-1* loss-of-function (Knock-Out) embryos express ectopic *repo* in hemocytes. (A) Immunstaining of *wt* and *miR-1* loss-of-function (LOF) embryos with anti-Srp antibody in red and anti-Repo in blue. Embryos were fixed at stage 15 of their development. The top panels represent a z-projection of the embryo and the bottom ones are confocal sections. Arrows indicate cells positive for both Repo and Srp. **(B)** Quantification of the number of Repo+Srp positive cells per embryo. Heterozygous (- / +) and homozygous (- / -) mutants were assessed against wild-type. At least 8 embryos were evaluated for the statistical analysis, using one way ANOVA. **: $p < 0.01$. Error bars represent standard error.

5.2.2 Adult glial *repo* expression is constantly necessary for CNS homeostasis

5.2.2.1 *repo* downregulation in adult glia reduces lifespan and motility

miR-1 downregulates *repo* in adult glial cells, and although *repo* co-expression alone alongside *miR-1* did not fully rescue the lifespan phenotype, the small effect shown by *Repo#9* left me still wondering whether adult *repo* expression was still important and could participate to the lifespan phenotype observed with *miR-1*. This time, an RNAi strategy was used to specifically downregulate *repo*, only at the adult stage using temperature sensitive Gal80 to inhibit Gal4 during development and then shift the flies at 29°C (from 3 to 5 days post eclosure onwards).

The lifespan of Repo Knock-Down (KD) flies was dramatically reduced using two different RNAi lines, coming from two different RNAi collections (VDRC for RNAi 1 (GD) and Bloomington for RNAi 2 (TRiP)). The flies from the RNAi 1 line die within 10 days while the ones from the RNAi 2 line die within 15-20 days (**Figure 5.4A**). In addition, the Repo-RNAi 1 flies exhibit strong motor defects from 7 days and become paralyzed by day 8-9 just prior death as assessed by climbing assay (**Figure 5.4B**). The Repo-RNAi 2 flies present a stronger difference between males and females compared to the Repo-RNAi 1 flies, which die within 2 days after the first motor defects for both sexes. The Repo-RNAi 2 males start to have motor defects by day 8 (**Figure 5.4B**, lower panel) and die by day 10-11 (data not shown). The females start to have motor defects by day 11 and are almost paralyzed by day 12 (**Figure 5.4B**, top right graph) but take longer to die as seen in **Figure 5.4A**. Thus, the onset of the motor defects correlates with the lifespan phenotypes.

The specific adult knock-down of *repo* was then assessed by western blot. When the flies are kept at 18°C, Gal80 inhibits Gal4 activity, thus *repo* is not downregulated and the motility and the lifespan are not affected (**Figure 5.4B** and **C** and **Figure 5.5A**). However, after only 3 days spent at 29°C to allow the siRNA expression and the downregulation of *repo*, a very strong reduction of Repo protein level was already observed, confirming the

efficiency of the RNAi (**Figure 5.4C**). It also demonstrates that Repo protein has a very fast turn over since within 3 days of activation of the RNAi, almost all the already produced proteins are not detectable anymore by western blot. The efficiency of the Repo-RNAi 2, giving a slightly weaker lifespan and motor phenotypes, was also evaluated. After 7 days spent at 29°C, while the RNAi 1 was fully downregulating *repo* expression, the RNAi 2 was less efficient, at least in females (**Figure 5.4E**). The Repo protein levels of males were not assessed. It is thus possible that knock-down is stronger in males than in females, which could explain the delay in the onset of the phenotypes. However, this weaker knock-down is still having a strong effect on both lifespan and climbing abilities (**Figure 5.4A** and **B**, right panel). These results show that a partial reduction of Repo protein level in adult glial cells has a very important impact on fly viability. Thus, the regulation of *repo* expression level in glial cells seems to be a critical issue for the CNS homeostasis.

In order to rescue the phenotype, a *UAS-Repo* line was co-overexpressed beside the RNAi. This line is sensitive to RNAi strategy, being a wild-type version of *repo*. However it is possible that it could counteract the effect by buffering the RNAi and allowing sufficient Repo protein level to get a rescue. The lifespan of these "rescue" flies was assessed in the first time. The rescue experiment by co-overexpressing *repo* beside the RNAi had a weak but significant effect, extending the lifespan by 5 days, starting from a lifespan of 10 days (**Figure 5.4D**). This "rescue" experiment displays actually a stronger phenotype in terms of short lifespan than using the Repo-RNAi 2 line. The levels of Repo protein were then assessed at different time point to understand the mild rescue of the co-overexpression of *repo*. After 3 days of expression at 29°C, the protein level in the "rescue" set-up is weak (**Figure 5.4E**). This result shows that the RNAi expression is strong enough to downregulate a very high level of *repo* expression. After 7 days at 29°C, there is still a residual level of Repo protein in the "rescue" condition, meaning that the *UAS-Repo* line is able to constantly counteract the RNAi 1 effect, even though it is to a very low extent (**Figure 5.4E**). The low levels of Repo protein in the rescue experiment, being even lower than the RNAi 2 knock-down alone can explain why the flies in this set-up live only 3-5 days more than the Repo-RNAi 1 flies compared to the 5-10 days difference between Repo-RNAi 1 and Repo-RNAi 2 and establish a good correlation between the levels of Repo

protein and the fly lifespan and the onset of the motor defects. In addition, the co-overexpression of *Repo#9* line beside the Repo-RNAi 1 did not rescue the lifespan phenotype (data not shown). This result might suggest that the *Repo#9* line is not as strong as the *UAS-Repo* and gives even more relevance to the small rescue of miR-1. Altogether, these results point again toward the fact that a tight threshold of Repo protein expression in glial cells is needed to maintain a viable organism. Since Repo-RNAi 1 was stronger than the Repo-RNAi 2 line and was downregulating fully the protein level, the rest of the study will be carried on with the Repo-RNAi 1 (GD from VDRC collection).

In order to investigate further the motor defects, I took advantage of *Tsh-Gal80*. This Gal4 inhibitor is known to be expressed in the VNC but not in the head and is broadly used to study the effects of motor neurons or generally VNC specific function (Davis et al. 2014). The Gal4 inhibition in glial cells of the VNC was first assessed as it was only tested for the Gal4 inhibition in neurons in the past. While the expression of Repo-RNAi downregulates the expression of *repo* in the entire nervous system using *Repo-Gal4* driver, the use of *Tsh-Gal80* inhibits the Gal4 activity in the VNC and rescues *repo* expression, at least partially since immunofluorescence cannot be used for accurate levels of expression (**Figure 5.4F**, arrows). Interestingly this also happens in the lamina glia of the optic lobe (**Figure 5.4F**, arrowhead). The motor defects observed previously were assessed again using *Tsh-Gal80*. The rescue of the *repo* expression only in the VNC is not enough to rescue the climbing defects since after 7 days at 29°C there is no significant difference between flies with or without *Tsh-Gal80* (**Figure 5.4F**, right panel). According to the previous western blot results using different RNAi lines, the more *repo* is downregulated, the earlier and more dramatic is the phenotype. It means that even though the levels of Repo protein in the VNC with *Tsh-Gal80* were not to wild-type levels, the motor defects would be at least delayed from the 7 days if the VNC was the only motor system responsible. Since it is not delayed, as the motor defects appear at the same time, it strongly suggests that the central brain is mostly responsible of the motor impairment. However, it is possible that the effect of the RNAi on the motor defects could come from the downregulation of *repo* in the PNS such as the sensory organs.

I next wondered whether some specific subpopulations of glial cells were responsible for the shortening of the lifespan, or at least would contribute to it. Similarly to the study done in the Chapter III, different Gal4 drivers were used. This time, they were combined with a ubiquitously expressed Gal80 temperature sensitive since the knock-down of *repo* during development leads to embryonic lethality or if expressed post embryogenesis, to the death at L1 larval stage, with the larvae being almost paralyzed before dying (data not shown). Once adult, the flies were transferred to 29°C to express the RNAi against *repo*. Contrary to what was observed when *polyQ-Atro* was expressed in different subsets of glial cells, only *dEAAT1-Gal4* driver expressing Repo-RNAi 1 decreased significantly the lifespan with a Chi square of 77 compared to less than 10 for the others (**Figure 5.4G**). And it has been previously established that when lifespans were done using 60 flies per condition (since the Chi square value is linked to the number of flies assessed), a Chi square smaller than 10 is irrelevant as observed also often between genetically identical control populations. Compared to the effect of *polyQ-Atro* expression in those same subpopulations of glial cells, it is quite clear that the phenotypes are different. In the Chapter III, with the *polyQ-Atro* expression, *dEAAT1-Gal4* had a phenotype very close to the one of *Repo-Gal4*, having very similar median survival (15-17 days for *Repo-Gal4>polyQ-Atro* compared to 20 days for *dEAAT1-Gal4>polyQ-Atro*), indicating that the *dEAAT1* positive cells were in majority responsible of the effects. However, using the same drivers to knock-down *repo*, *Repo-Gal4* driver has a much stronger phenotype than the one of *dEAAT1-Gal4*, having a median survival of 8-9 days compared to 25 days for *dEAAT1-Gal4>Repo-RNAi 1* (**Figure 5.4G**). This difference indicates that in the case of *repo* downregulation, the *dEAAT1* positive cells do not account for most of the effect on lifespan as it is the case for *polyQ Atro*. More specific drivers covering subtypes in *dEAAT1-Gal4* positive cells, such as *Alrm-Gal4* or *NP2222-Gal4*, were having significant effect when expressing *polyQ-Atro* with Chi squares between 30 and 60. The same drivers for Repo-RNAi 1 have respectively a Chi square of 7 and 10 (**Figure 5.4G**). These results indicate that the downregulation of *repo* in these specific subtypes of glia has a much weaker impact on *Drosophila* viability than the expression of a toxic polyQ protein in the same cells. The strength of the phenotype using *dEAAT1-Gal4* driver expressing Repo-RNAi 1 confirms this observation. When using *Moody-Gal4* to express Repo-RNAi, which had a Chi square of 4 when expressing *polyQ-*

Atro, the phenotype was not significant and having a Chi square of 2 for the expression of Repo-RNAi 1. Another driver, *Gliotactin-Gal4*, could be used for BBB glia. It has been shown in the Chapter III that it has stronger effect than *Moody-Gal4* for *polyQ-Atro* expression. Unfortunately, a technical problem with the incubator arose while the lifespan using this driver was being done and lack of time did not allow me to repeat it. It would be interesting to see whether a stronger BBB driver could trigger lifespan defect by specific knock-down of *repo*. This could link BBB defects to the reduction of the lifespan and/or motor impairments.

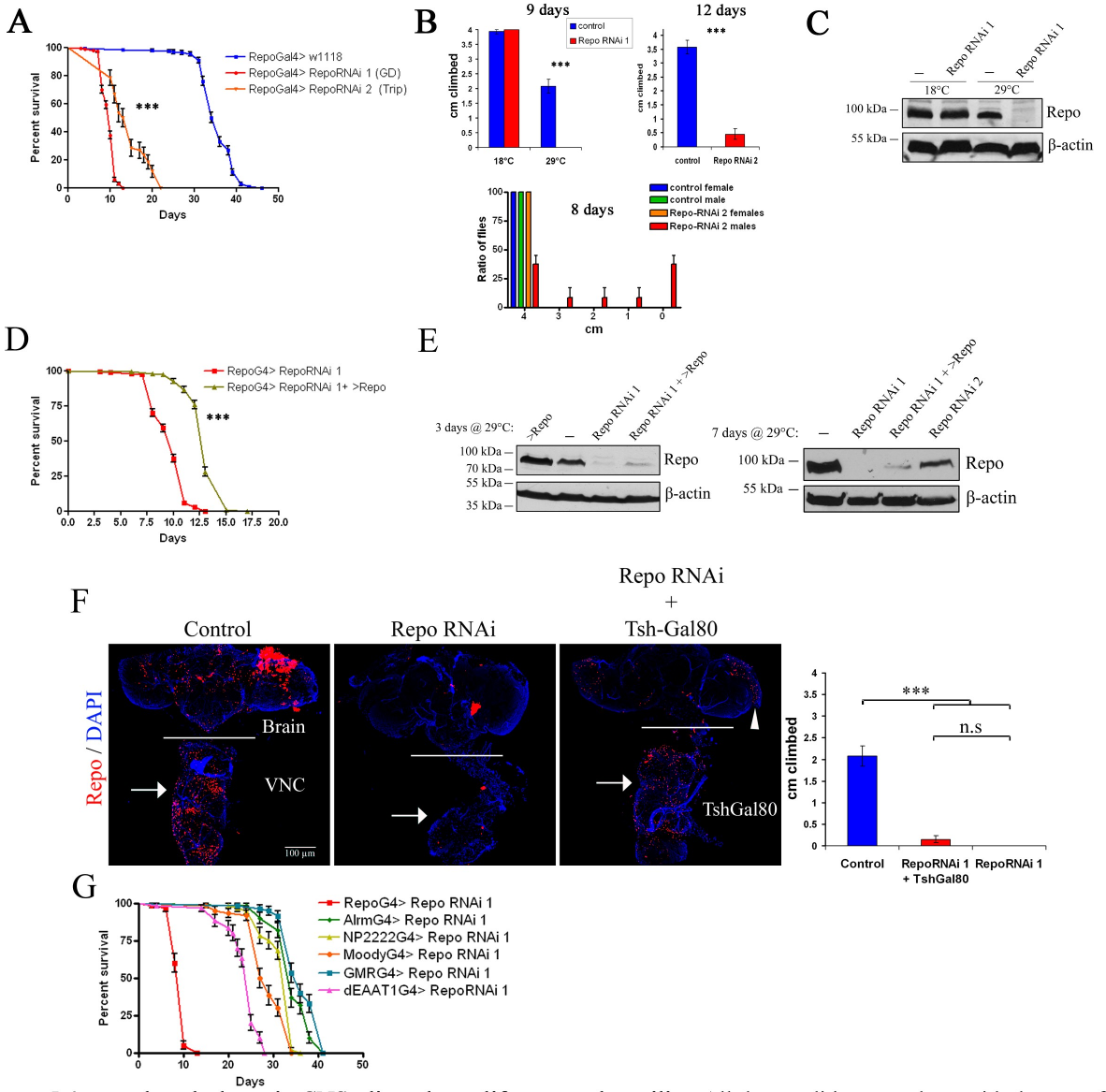


Figure 5.4: *repo* knock-down in CNS glia reduces lifespan and motility. All the conditions are done with the use of *UbiGal80^{ts}*. *w^{1118}* is used as control. **(A)** Lifespan of flies expressing two different RNAi lines against *Repo* in adult ('1' for GD from VDRC and '2' for TRiP from Bloomington collections). **(B)** Climbing assay of both RNAi lines 1 and 2 at different time point, respectively 9 days and 12 days prior to death when being paralyzed. The genotypes are similar to **(A)**. 18°C condition is to control for Gal4 inactivity with no RNAi expression. The bottom graph shows the ratio of flies performing for each score assessed during the climbing assay. The males of the Repo-RNAi 2 line start to show defects at 8 days while the females are not significantly different from control. The males RNAi were compared to males from control using the average climbing by a t-test and was statistically significant p=0.004 **(C)** Corresponding Western blot of Repo to the time points of the climbing assay. **(D)** Lifespan of the rescue of RepoRNAi 1 using a transgene overexpressing wt *Repo*. **(E)** Western blot of flies sacrificed after respectively 3 or 7 days at 29°C, allowing the expression of the different transgenes. Note that Repo-RNAi 2 has a weaker downregulation of *Repo*, corresponding to a milder decrease of the lifespan. **(F)** Pictures showing the effect of the inhibition of Gal4 activity in the VNC using *TshGal80*. Right panel: Climbing assay after 9 days at 29°C showing that the specific expression of Repo-RNAi in the brain is enough to trigger motor defects. Arrows indicate the VNC and the arrowhead shows the expression of *Repo* in the lamina of the optic lobe in the *TshGal80* condition. **(G)** Lifespan of flies expressing Repo-RNAi 1 in different subset of glial cells using specific Gal4 drivers (described in Chapter III). All lifespans have been done using more than 60 flies. Statistical analysis was done as previously described. More than 5-15 flies were used in each condition for the climbing assay. Each climbing were done in triplicates. T-test was used for statistical analysis. For both lifespan and climbing assay, ***: $p < 0.001$. Error bars represent standard error.

5.2.2.2 Adult *repo* expression level is tightly regulated via a potential positive feedback loop

The knock-down of *repo* is already dramatic after 3 days of RNAi expression. Using the possibility to turn on and off transgene expression by shifting temperatures from 18°C to 29°C but also 29°C to 18°C, I asked whether a high level of Repo protein is constantly needed in glia in order to maintain *Drosophila* viability. The hypothesis is that the knock-down of r*epo* during a short period of time would be enough to trigger fly death. The flies are grown at 18°C. Once adult, they are shifted to 29°C for only 3 days and then put back at 18°C in order to block the expression of any transgene. In these conditions, when the RNAi against *repo* is expressed only during 3 days, the lifespan is still dramatically reduced. Indeed, whereas the control flies have more than 80% alive flies after 50 days, the Repo-RNAi ones have a median survival of 16 days and are all dead by 20 days (**Figure 5.5A**, lifespan labelled "**3d**"). The median survival is only delayed by a week compared to flies constantly exposed to 29°C. This difference could actually be explained by the fact that the entire organism is slowed down at 18°C, which would then slow down also the moment of the death. This result shows a completely different mechanism to *polyQ-Atro* build up process, for which a mere 3 days expression does not affect *Drosophila* lifespan (data not shown). In order to control that flies kept at 18°C are not affected by a leakage of Repo-RNAi or the insertion of the transgene, a lifespan done entirely at 18°C was done. There was no difference between the control flies and the Repo-RNAi ones (**Figure 5.5A**, lifespan labelled by "**18C**"). The rescue experiment was then done in order to see if the small levels of Repo protein observed after 3 days at 29°C would be enough to maintain the organism alive longer. The flies co-expressing Repo-RNAi and *UAS-Repo* at the same time were partly rescued, with a median survival of 34 days compared to 16 days without the overexpression of *repo*. 15% of the flies were actually still alive after 50 days at 18°C. Moreover, motor capacity was also assessed after 7 days at 18°C, post 3 days at 29°C. As seen in **Figure 5.5B**, the co-expression of a *repo* copy is also enough to significantly rescue motor defects. This robust rescue of almost 20 days (compared to the 3 days observed at 29°C) is unlikely to represent only a slowdown of the organism at 18°C. It is thus possible

that the residual amount of Repo protein, present after 3 days when *UAS-Repo* transgene is present, is enough to maintain some critical functions of the glial cells to keep the fly alive for longer.

As mentioned in the Chapter I, it has been suggested that *repo* could act as a positive feedback loop on its own promoter during embryogenesis, but could be specific to certain conditions, like the presence of other activators or repressors. With the Repo-RNAi flies dying so quickly even after only 3 days spent at 29°C and then shifted at 18°C, I wondered what the protein levels were after few days spent at 18°C. The Repo protein levels at the same time point as the climbing assay were analysed by western blot. At this time point, the Repo-RNAi flies are about to start dying and have motor defects while the rescued flies (Repo-RNAi 1 + *UAS-repo*) perform better at climbing and are still 10 days ahead of the first flies dying (**Figure 5.5A**). In the case of the overexpression of *repo* only, the protein levels are not increased anymore compared to 3 days spent at 29°C (**Figure 5.5C** compared to **Figure 5.4E**, left panel). This result suggests that the Gal4 activity is abolished within few days after the shift of temperature and that the fast turnover of the Repo protein allows a tight regulation to maintain a specific level. However, in the Repo KD condition, the protein level was not back to normal and was still dramatically decreased, being not detectable by western blot analysis (**Figure 5.5C**). For the rescue experiment, the level of Repo protein was still strongly decreased, as it is the case already after 3 days and 7 days at 29°C (**Figure 5.5C**). This result points partly toward a regulation by positive feedback loop as the Repo protein needs to be present, or expressed to a certain level, in order to get the endogenous protein expression back to its normal level. It is also possible that a previously accumulated pool of siRNA is still present, blocking the production of newly formed *repo* mRNA, which keeps Repo protein level down for 7 days since double strand siRNA can be rather stable in a cell for up to 3 days (Bosch et al. 2016) (Ameres et al. 2013) (Bartlett et al. 2006). However, after 7 days at 29°C in the rescue condition, there is also still a residual amount of Repo protein, indicating that the RNAi knock-down capacity has reached a maximum saturation. Furthermore, in the case of the 3 days at 29°C and then shift to 18°C, the small amount of protein present in the rescue is likely to come from mRNA produced by the endogenous *repo* gene, since the *UAS-Repo* transgene is not active at 18°C. It shows

that the small residual amount present after 3 days at 29°C is able to maintain at least a low level of protein production from new transcription of the endogenous *repo* gene. In order to confirm this hypothesis and to rule out the effect of potential RNAi during few days at 18°C, Repo protein levels were assessed in both control and "rescue" flies that spent 3 days at 29°C and 20 days at 18°C. Since both RNAi and exogenous Repo protein are not produced anymore once at 18°C, the Repo protein present in the western blot will come from endogenous *repo* expression. Also, if after 7 days at 18°C the low levels of *repo* expression were due to the presence of a pool of siRNA accumulated at 29°C, this pool is likely to decrease over time, either by degradation or by its use to degrade newly produced *repo* mRNA. As a result, the protein level should increase as the siRNA pool would decrease. However, after 20 days at 18°C, the Repo protein level is still very low in the rescue condition, comparable to the one after 7 days at 18°C **(Figure 5.5D**). Thus, it is unlikely that the low level of Repo protein observed after 20 days at 18°C is due to a continuous knock-down of endogenous *repo* expression due to a pool of siRNA present after 3 days at 29°C. This result strengthen the hypothesis that a low level of *repo* expression is able to constantly maintain a low level of Repo protein but is not able to increase its expression level to go back to wild-type level. Surprisingly, the loading of all the conditions together on the same western blot showed that the Repo protein level after 3 days at 29°C in both control and rescue conditions are reproducibly higher than the other ones (**Figure 5.5D**). It is possible that *repo* expression increases as a result of a heat shock response of glial cells before going back to normal levels after few days, as seen in the decrease of Repo protein level after 7 days at 29°C. It is also possible that there is an aging effect of *repo* expression. At 18°C, 7 days or 20 days are still early time points in *Drosophila* lifespan, which can survive more than 3 months at this temperature. Later time points would be needed to confirm this hypothesis. In the specific case of the *repo* rescue, there is further possibility that the decrease in Repo protein level observed between 3 days at 29°C and 3 days 29°C + 7 days 18°C could come from the effect of siRNA already produced before the shift of temperature, downregulating *repo* expression for few days before being all degraded or used. Then the low level of *repo* expression would remain. It would be required to establish whether the decrease between 3 days at 29°C and 3 days

29°C + 7 days 18°C in the rescue condition is the same than the one obtained in the control condition to clarify if an additional effect is present with the RNAi.

In order to confirm a potential effect of *repo* on its own promoter, a *repo* reporter was used. This reporter is the coding sequence of a nuclear *GFP* (*nGFP*) downstream a 4.2kb portion of the *repo* promoter. A western blot analysis of the GFP signal after 7 days at 29°C was done, with or without Repo-RNAi. There is a clear and reproducible reduction of the GFP protein level compared to control (**Figure 5.5E**). The residual GFP protein in the Repo-RNAi condition could be mere perdurance since it is known that GFP is relatively stable within a cell, especially when localised to the nucleus. This result strongly suggests that to be activated, this portion of *repo* promoter needs the presence of Repo protein and vice versa that Repo protein downregulation could also be partly mediated by a loss of transcription from the *repo* promoter. Altogether, these results provide strong evidence that *repo* expression could act as a positive feedback loop in the adult *Drosophila* glial cells. However, the results from the western blot of the rescue (Repo-RNAi + >Repo) (**Figure 5.5C and D**) show that it might not only be a simple positive feedback loop and that some point of no-return is reached regarding the possibility of activating this promoter, behaving like a bistable switch. Indeed, the Repo protein level should be back up to normal if the presence of Repo protein was enough to activate its own expression. It is possible that a certain threshold is needed to bind to its own promoter, or other co-activating factors are present only when Repo protein is highly expressed or that in absence of Repo, its promoter is irreversibly shut off by possible epigenetic modifications.

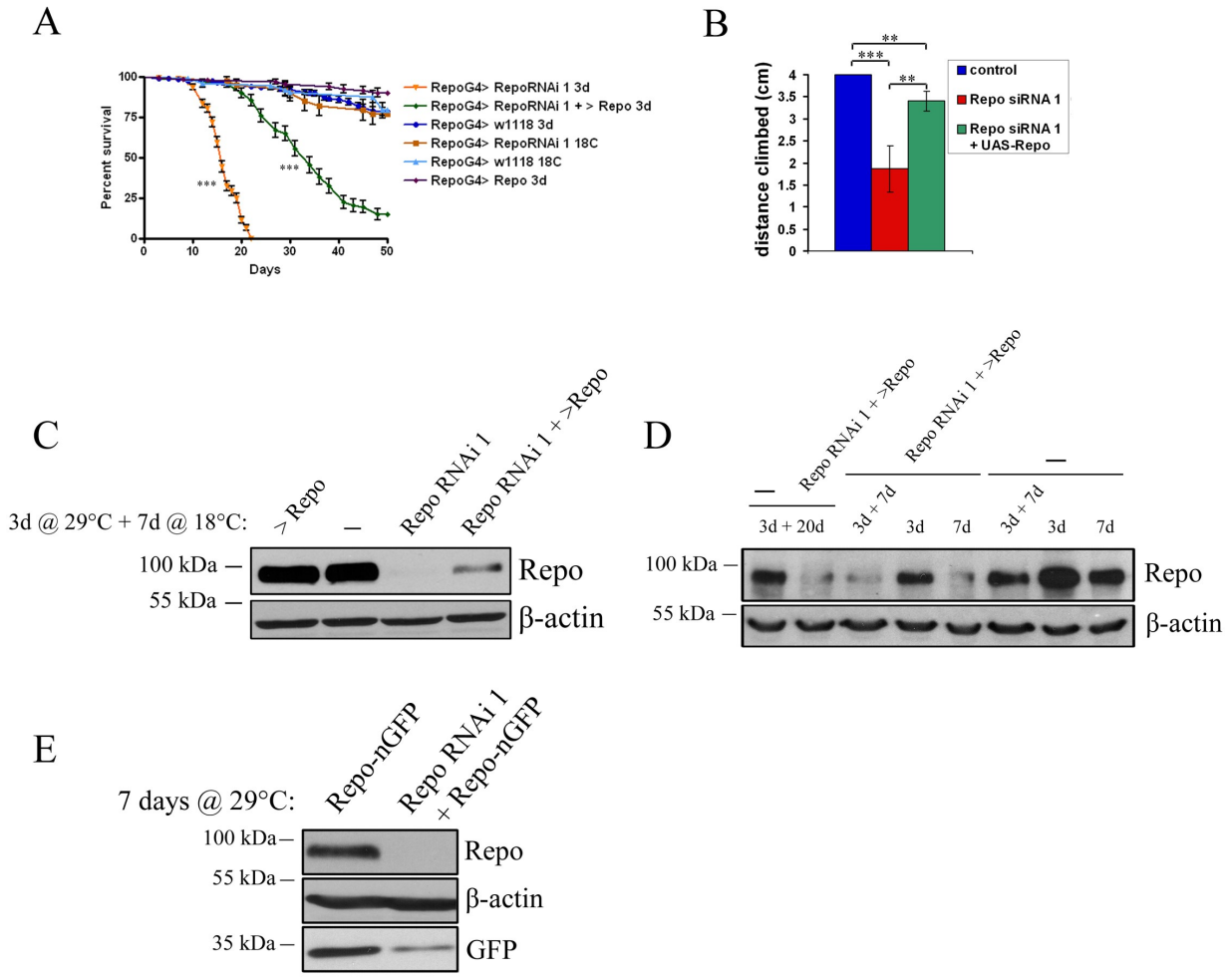


Figure 5.5: Repo protein is needed for its own expression suggesting a positive feedback-loop. All conditions are done with the use of *Repo-Gal4,UbiGal80^{ts}*. *w^{1118}* is used as control. **(A)** Lifespan of flies expressing different transgenes during 3 days at 29°C and then transferred at 18°C to block Gal4 activity (labelled 3d). The other lifespans labelled 18C correspond to flies that have only been exposed to 18°C thus never expressing any transgenes. **(B)** Climbing assay of flies exposed 3 days at 29°C and then 7 days at 18°C, corresponding to the time points of the Western blot in (C). **(C)** Western blot of flies sacrificed after 3 days at 29°C and 7 days at 18°C. **(D)** Western blot of control flies or flies expressing Repo-RNAi 1 + UAS-Repo sacrificed at different time point, with 3 days at 29°C + 20 days at 18°C in the first two lanes to control if the *repo* expression was coming back over time. **(E)** Western blot using anti-GFP Ab to evaluate *Repo-nGFP* transgene expression levels after 7 days at 29°C. All lifespans have been done using more than 110 flies. Statistical analysis was done as previously described. More than 15 flies were used in each condition for the climbing assay. Each climbing were done in triplicates. One way ANOVA was used for statistical analysis. ***: $p < 0.001$ and **: $p < 0.01$. Error bars represent standard. error.

5.2.3 *repo* downregulation does not increase the sensitivity to oxidative stress

The expression of *repo* in adult glia is important at organism level, since the viability is dramatically reduced when it is knocked-down. Moreover, in addition to lethality, it impairs motor functions, surely coming from defects in neuronal activity since neurons control behaviour directly and glial cells interact strongly with neurons and regulate their activity, especially at the synaptic level. After investigating *repo* regulation at the molecular level, I wanted to understand what is happening at the organism level and try to characterize the possible causes of death. The glial cells providing a trophic support to the neurons and clearing neurotransmitters at the synapse, it is possible that if they fail in some of these functions, this can trigger neuronal damage, by oxidative stress for instance. Potential damage of the mitochondria can lead to a certain vulnerability to further stress, when the system is more challenged. And oxidative stress is known to reduce lifespan in flies (Fleming et al. 1992, Le Bourg 2001). A way to assess an increase in the sensitivity to oxidative stress is to feed the flies with paraquat. The paraquat generates free radicals and impairs mitochondrial functions (Hosamani et al. 2013). This molecule is generally used to assess resistance or increased sensitivity to oxidative stress.

After spending 3 days at 29°C as adult to activate the RNAi expression, the flies are transferred to 18°C into vials with paraquat (see Material & Methods, Chapter II) and the death was recorded several times per day and the flies were maintained at this temperature. According to the results from the previous chapter, *repo* remains downregulated after the 3 days at 29°C. However, no difference was observed between wild-type and Repo-RNAi 1 flies (**Figure 5.6**). The flies in this set up start to have motor defects at 7-8 days, similarly to full 29°C set up. Only the start of death event is delayed by a week. The flies remained 4 days in paraquat at 18°C, after spending 3 days at 29°C. This window for the experiment has been selected in order to monitor early phenotypic events possibly triggered by an exposure to strong oxidative stress. At later time point, it would be difficult to distinguish death events due to the paraquat or the fly death happening without it. This result indicates that there is no increased sensitivity to oxidative stress early on that could be responsible

for the neuronal dysfunction and death later on. However, it does not rule out increased sensitivity to oxidative stress later on, closer to time of death but cannot be assessed reliably given the fast decay of Repo KD flies.

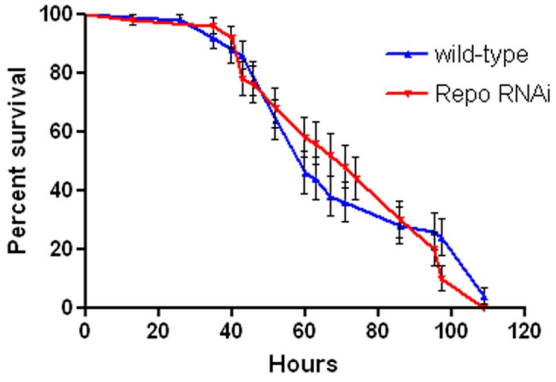


Figure 5.6: *repo* downregulation does not increase the sensitivity to oxydative stress in early stage. The genotypes are: *Repo-Gal4,UbiGal80^{ts}* crossed to either *w^{1118}* or *UAS-Repo-RNAi 1.* The 3 first days are at 29C and then the flies are shifted at 18C. The statistical analysis between both genotypes has been done as previously mentioned, using GraphPad Prism. 25 flies were assessed for each condition. There is no statistical differences. Error bars represent standard error.

5.2.4 *repo* knock-down in adult glia does not trigger cell death or change of fate contrary to developmental stages

It has been shown by our collaborators that in the embryonic VNC, the downregulation of *repo* could lead to the expression of neuronal marker such as *Elav* by presumptive glia, using the *Repo-nGFP* as glia reporter. (Trebuchet et al. 2016, manuscript in preparation). This confirms that *repo* is able to repress neuronal fate determination in glia. According to these results, and the fact that *repo* is a cell fate determinant, still expressed in adult differentiated cells, I asked whether it was possible that even in the adult fly, glial cells could have a certain flexibility in their identity that would allow them to adapt to some situations encountered throughout the life (CNS injury for instance). A switch to neuronal fate by glia would be surely a major disruption for the fly CNS and could lead to the motor defects and early lethality observed. Using the same set up as in the embryo, I co-stained brains and VNC of adult *Drosophila* with anti-GFP and anti-Elav antibodies, in the presence or not of Repo-RNAi. However, in Repo KD flies, no cells have been found positive for both GFP and Elav at the same time, either in the brain or in the VNC (**Figure 5.7**). This negative result goes against a potential role of *repo* in repressing neuronal fate in adult glia. However, we cannot rule out this possibility as the time course in the observation was few hours in embryos and 7 days in the adult. It is possible that the GFP signal from *repo* cells is already not detectable from the cells that would have changed fate.

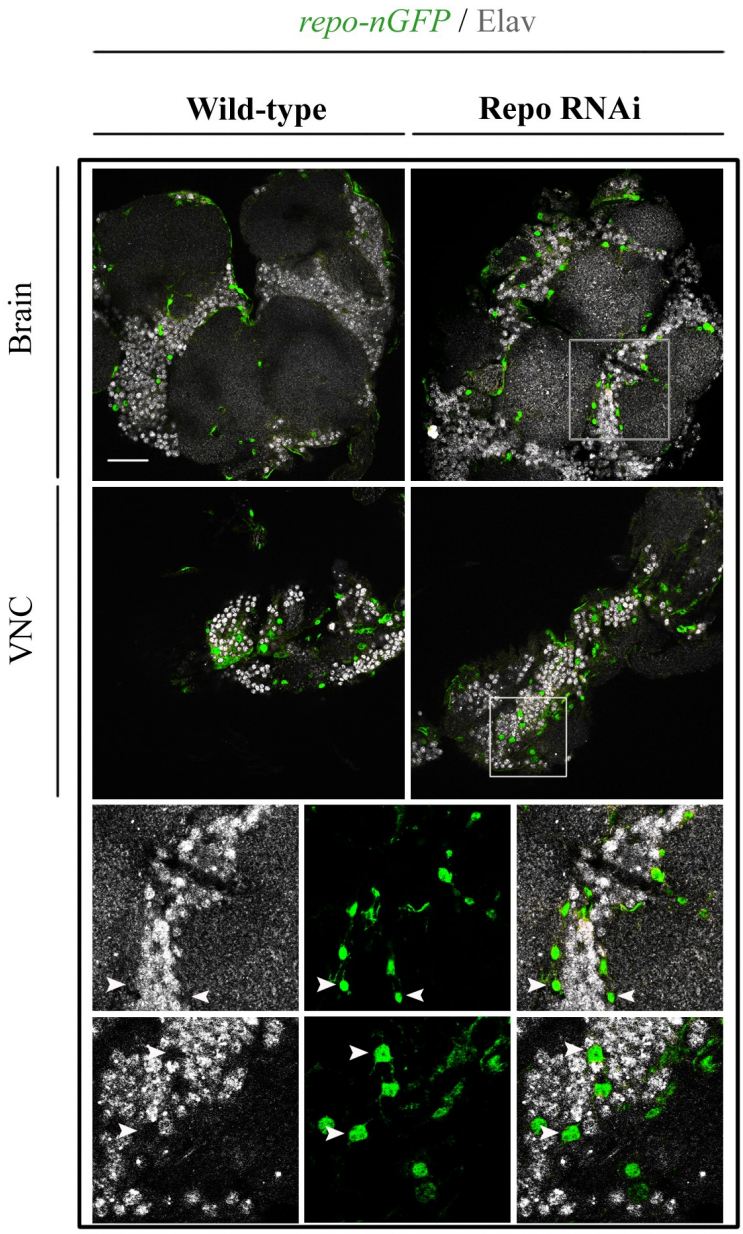


Figure 5.7: *repo* does not repress neuronal cell fate in adult glia. The genotypes are: *Repo-Gal4,UbiGal80^{ts} x Repo-nGFP +/- UAS-Repo-RNAi 1.* Immunolabelling of wild-type and *Repo* knock-down in adult brains and Ventral Nerve Cord (VNC) (abdominal segment). The flies were aged 7 days at 29C until they reach paralysis state. They are then dissected and stained with anti-GFP and anti-Elav antibodies. *Repo-nGFP* transgene was used to label presumptive glial cells. GFP (green) and Elav (grey). Scale bar: 30um. The lower panels represent higher magnification of Repo RNAi condition in the brain (top panels) and in the VNC (lower panels). Arrowheads indicate glial cell nGFP, which never colocalizes with Elav staining.

To verify this hypothesis, the number of glial cells per specific areas of the brain was assessed, using the *Repo-nGFP* marker. Counting GFP positive nuclei both in the central brain or optic lobes, no significant difference was observed between wild type and Repo-RNAi conditions (**Figure 5.8A**, the quantifications represent the counting of green cells in the full brain using ImageJ software. The images represent the optic lobes as an example of methodology). This result goes against the hypothesis that glial cells would have transformed into neurons and lost their GFP signal before the flies are sacrificed and stained. In this case we would have observed a drop in the number of glial cells present in the brain or an increase in $elav^{+}$ cells. It is noteworthy that the immunostaining of nGFP seems contradictory with the western blot of the **Figure 5.5E**. However, in this case, the settings of the microscope were not calibrated on the control samples and thus no quantitative results can be assumed from the pictures on the level of GFP even though it seems strongly decreased from the western blot. In order to be more reliable and independent of the reporter (dependent on Repo protein presence), an irreversible labelling of the glial cells would allow a better counting to identify a drop in the cell number.

In *repo* hypomorph mutants, it was shown that there were signs of neuronal apoptosis and degeneration. Glial cells being responsible of neuronal maintenance, by providing energy and trophic support, the potential cell death was evaluated by TUNEL assay, which stains for DNA breaks. No significant difference was observed between control and Repo-RNAi flies, both of them having no more than 10 cells positive (**Figure 5.8B**). A sample treated with DNAse was done to confirm that the enzymatic activity of the assay has worked. It suggests that the fly death is not due to any cell loss, even though a time course for the assay would be needed to evaluate previous cell loss. In the case of the hypomorph mutant used in the past, the mutation of *repo* had its effect from embryo through all developmental stages. As a result, the glial cells in the optic lobe were misplaced from early development to adult, which could have led to more severe phenotypes as the glia would have failed to provide their correct supports from developmental stages. In the case of specific adult knock-down, the glial cells are correctly located in the CNS and their functions during the developmental stages were not affected. This could explain the difference observed between the previous study and the adult phenotype.

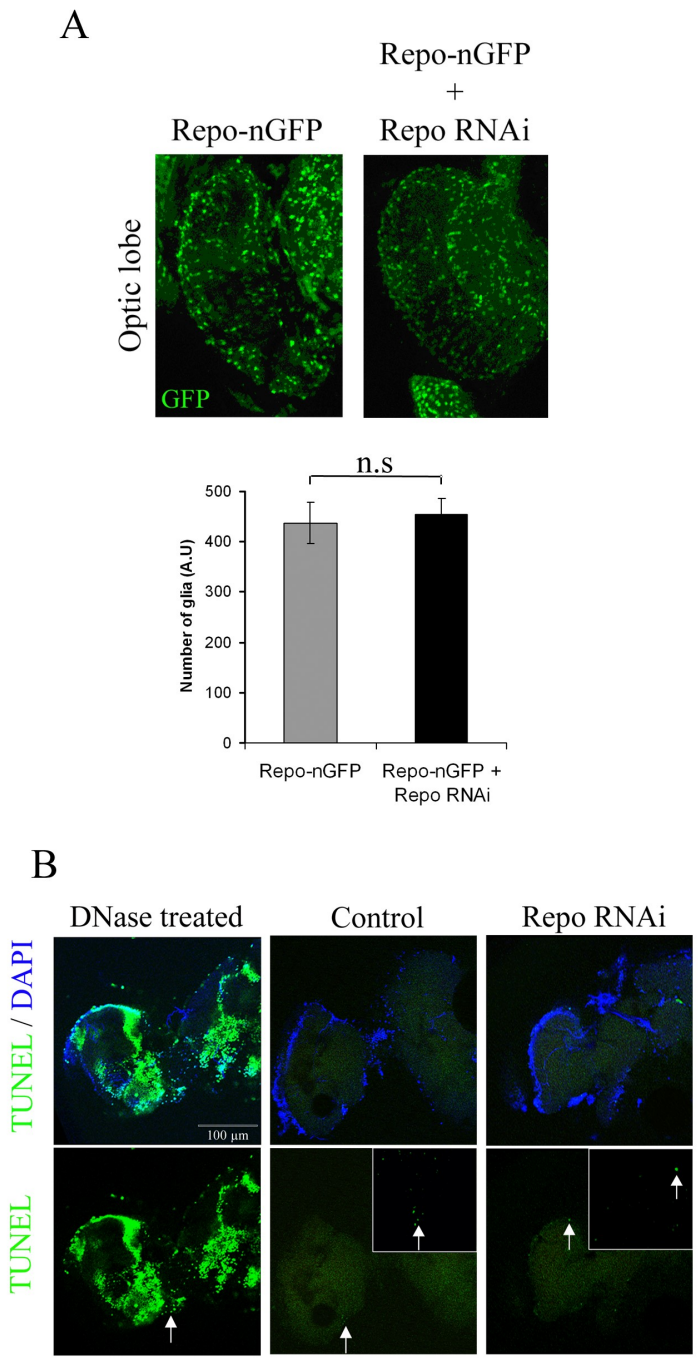


Figure 5.8: *repo* is not necessary for glia survival and does not trigger neuronal cell death. The genotypes in all conditions are: *Repo-Gal4,UbiGal80^{ts}* crossed with *Repo-nGFP* +/- *UAS-Repo-RNAi 1*. **(A)** The same *Repo-nGFP* reporter has been used to count the number of glial cells per brain in control or Repo-RNAi condition after 7 days at 29°C. As represented by pictures of optic lobes and the quantification of 8 brains, there is no difference in the number of cells expressing *Repo-nGFP* between control and Repo-RNAi flies. The statistical analysis was done using t-test. **(B)** TUNEL assay. In green are the fluorescent newly integrated dNTPs to DNA breaks. The DNase treated condition serves as positive control for the reaction. The arrows show TUNEL positive cells. No difference was observed between control and Repo-

5.2.5 *repo* regulates the expression of glial-specific genes and functions in adult stage

The previous results have shown that the different phenotypes observed (decrease of lifespan and motor defects) do not seem to come from a loss of cells in the CNS. However, it is possible that potential cell loss in the PNS could have caused these phenotypes, in particular the motor defects. Glial cells interact strongly with neurons at multiple levels such as synaptic level, axonal ensheathment or energy support. Dysfunction of neuronal activity or failure to maintain normal cell homeostasis in the CNS can lead to the death prior any sign of degeneration.

5.2.5.1 *repo* controls the activation of specific genes in adult glia

Since there were no signs of cell death or fate change, I set out to test whether there were any changes at the molecular level which might result in functional alterations in glia. Using a ChIP assay on S2 cells, *repo* has been shown to potentially bind to several gene promoters, including glia-specific genes. It is has been demonstrated for some of them, including the *Glutamine synthetase 2* (*Gs2*), *dEAAT1* and *dEAAT2* genes, that *repo* null (-/-) mutant embryos did not express any of the above mentioned genes mRNA (Soustelle et al. 2002, Kerr et al. 2014). Since *repo* is involved in the activation of specific sets of genes during the terminal glial differentiation, it is possible that, being still expressed, its presence in adult glia is still required to maintain the expression of such genes. A list of some genes obtained by the ChIP, known to be expressed in *Drosophila* glia or in the Wg signalling pathway, has been published by Kerr et al. I decided to focus on some of those genes, based on their functions or previous established direct interactions: *Gs2* (expressed mostly in astrocyte and involved in glutamate recycling in glial cells), *dEAAT1* (Glutamate transporter that shows motor defects in both larvae and adult when downregulated (Rival et al. 2004)), *Gliotactin* and *loco* (involved in the establishment and maintenance of the BBB (Auld et al. 1995, Schwabe et al. 2005)), *wg* (secreted by glia at the larvae neuromuscular junction (NMJ) under the control of *repo* (Kerr et al. 2014)) and *Pointed* (known to co-

activate with *repo* some specific genes in glia such as *loco* (Yuasa 2003)). The only known *Drosophila* glial GABA transporter *Gat* was also added to this list of genes (Stork et al. 2014), based on the fact that it was very specific to astrocyte-like glia and likely to be under the control of a glia specific transcription factor. In order to see whether *repo* was able to control the expression levels of such genes in adult *Drosophila* brain, a qPCR was done at different time point, either in wild type or RNAi conditions. The two time points chosen were after 5 hours spent at 29°C, before any possible effect of the expression of the RNAi on *repo* expression but the flies were still subjected to this temperature to control for a potential increase in gene transcription due to a higher temperature, and after 7 days, when the flies start to have motor defects, 3 days prior to death, in order to maximize the chances to see potential changes in other gene mRNA levels. Two reference genes were used, *Gapdh* and *eIF4A*, previously used in a microarray study in the lab (Napoletano et al. 2011), being common control genes.

While after 5 hours at 29°C there are no significant changes in all the analysed genes, after 7 days at 29°C, several genes display a statistically significant decrease in their mRNA levels (**Figure 5.9A**). Indeed, *dEAAT1*, *Gat*, *Gs2* and *loco* have a significant reduction of their mRNA level and both *dEAAT1* and *Gat* are also significant post FDR corrections, with *Gs2* and *loco* having a P-value post FDR of 0.06 and 0.07 respectively. However, the other genes did not reach significance. It is possible that for a gene like *wg*, not only expressed in glial cells, the level of mRNA could be diluted by its expression from other cells since the qPCR is done on whole head samples. These four genes are involved either in neurotransmitter clearance and recycling or in BBB maintenance. Each of these functions has been shown to be important in motor functions or generally longevity (Kim et al. 2010), which correlates with the Repo-RNAi phenotypes. *Gat* being the gene with the strongest mRNA level reduction, its protein level was analysed by western blot, again after 7 days spent at 29°C. *Gat* is known to express three protein isoforms (Muthukumar et al. 2014). In the **Figure 5.9B**, the western blot analysis of *Gat* protein level shows that all three isoforms seem to be downregulated, though to a different level. While the middle isoform is strongly downregulated, the top one is only mildly decreased. The signal of the lower band is a lot weaker so it is more difficult to assess it, however, the signal appears to

fade in Repo-RNAi condition. These results go in the same direction than the qPCR data for *Gat*, which indicate that *repo* could control, at least indirectly, the activation of *Gat* transcription in adult *Drosophila* astrocyte-like glia. However, the question of a direct or indirect control remains open.

According to the qPCR data and the western blot for *Gat*, I asked whether the overexpression of each of these four genes would be able to at least partially rescue the lifespan phenotype of *repo* knock-down. However, none of them alone has been able to increase the lifespan, with the overexpression of *loco* decreasing it significantly even more ($p<0.001$) (**Figure 5.9C**). It is possible that, *Repo-Gal4* driver being a knock-in and Repo protein possibly acting on its own promoter, the *Gal4* expression becomes very low when Repo protein is absent. Thus the expression of other transgenes in parallel of Repo-RNAi could be blocked or not high enough to rescue such a strong phenotype. The only significant effect is an even further decrease of the lifespan by overexpressing *loco*. While rescuing a phenotype can be difficult if it is very strong or coming from different origins, an additive effect can be more easily seen. It has been shown that the overexpression of *loco*, similarly to its knock-down, could have detrimental effect on the BBB, indicating that a tight control of its expression level is required for its function in the BBB when interacting with different GPCR signalling molecules (Schwabe et al. 2005). Moreover, it was suggested that its overexpression could reduce the *Drosophila* lifespan (Lin et al. 2011). This last study was overexpressing *loco* using a ubiquitous driver, hiding a potential specific role of glial cells in these phenotypes. However, when overexpressed specifically in adult glial cells, it did not reduce the lifespan (**Figure 5.9D**).

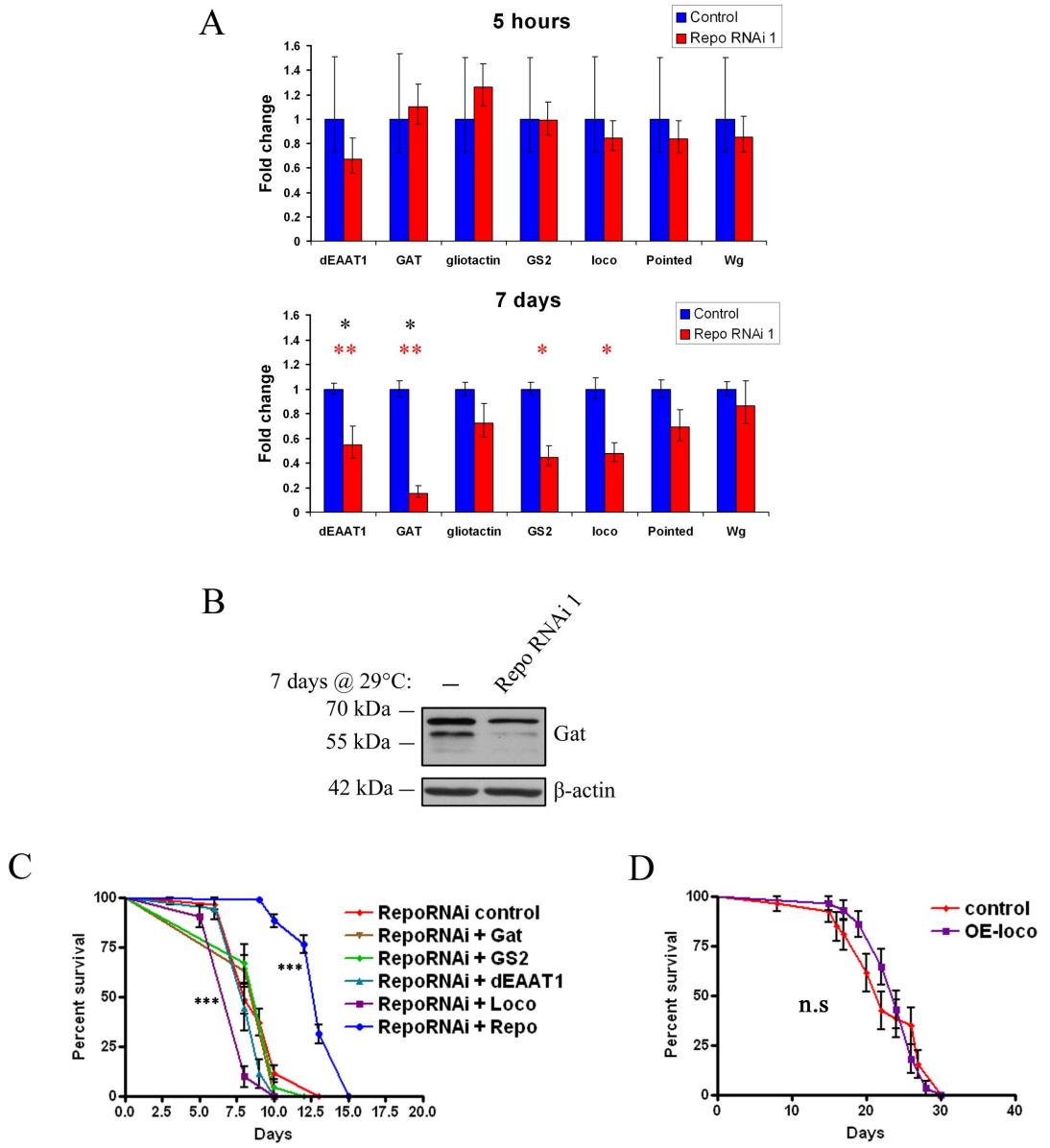


Figure 5.9: *repo* activates several glia-specific genes in adult *Drosophila*. In all the conditions, the driver used is: *Repo-Gal4,UbiGal80^{ts}*. *w^{1118}* is used as control. **(A)** qPCR results of potential direct *repo* target genes. After 5 hours spent at 29°C (reference time point before the RNAi can downregulate *Repo*), there is no statistical difference between control and Repo-RNAi flies. After 7 days at 29°C, several genes are downregulated in the absence of Repo protein. Statistical analysis was performed using DataAssist software. **: $p < 0.01$ and *: $p < 0.05$. Black stars represent post FDR correction. **(B)** Western blot of control and Repo-RNAi brains using same time points as for the qPCR, using anti-Gat antibody. Note that there are three bands, with the middle one the most affected. However, all three are consistantly downregulated **(C)** Lifespans of Repo-RNAi combined with several UAS-transgenes (overexpression) from the most downregulated genes in the qPCR experiment. **(D)** Lifespan of flies overexpressing only *loco* (*UAS-loco*) compared to control, without *Repo-RNAi*. There is no statistical difference. The analysis has been done as previously mentioned using GraphPad Prism. More than 20 flies were assessed per conditon. Note that the co-expression of Loco beside Repo-RNAi significantly decreases even further the lifespan (p=0.0008). *dEAAT1* co-expression has a p-value of p=0.06.

5.2.5.2 *repo* expression in adult glial cells maintain the BBB integrity

According to the previous results, especially the fact that *loco* overexpression was worsening the phenotype, I wondered whether a potential BBB impairment could be linked to the fly death and associated to the motor defects. By injecting a fluorescent dye in the fly thorax, the dye reaches the hemolymph and diffuses throughout the body (Bainton et al. 2005). If the *Drosophila* BBB is intact, the dye does not penetrate the brain and accumulates around it. However, if there is BBB impairment, the dye can leak into the brain. By confocal microscopy, the presence of the dye inside the brain can be assessed to identify potential BBB leakage. All the four conditions (wild-type, Repo-RNAi 1, Repo-RNAi 1 + UAS-Repo and Repo-RNAi 2) were assessed first after 8 days at 29°C, the dye being injected the day before, when the Repo-RNAi 1 flies do not move. However, at this time point the flies from the other genotypes, wild-type, Repo-RNAi 2 and Repo-RNAi 1 + UAS-Repo, do not display motor defects yet, having their median survival delayed by few days.

The analysis of the BBB leakage was done by evaluating the strength of the leakage. If the dye is kept outside, it is considered intact. If the dye is present in specific areas, close to the edge of the brain, it is considered mild penetration and if it is throughout the brain, it is a strong penetration. The wild-type flies have their BBB intact in most of the cases, only 3 out of 18 brains had mild dye penetration (**Figure 5.10**). In the case of a very strong knock-down of *repo*, using the Repo-RNAi 1, 14 out of 16 brains had dye penetration and in most of the cases it was throughout the entire brain, with a strong penetration (**Figure 5.10**). When *repo* is overexpressed beside Repo-RNAi 1 (RNAi 1 + >Repo), which delayed the death by only 3 days, 6 out of 8 brains were positive for the dye penetration (**Figure 5.10**). However, half of the positive ones were mild penetration of the dye, similarly to wild-type positive cases. When the Repo-RNAi 2 is used to knock-down *repo*, which delay the death by a week in females and has a weaker knock-down of Repo protein after 7 days at 29°C, only 3 out of 10 brains were positive for the Dextran dye (**Figure 5.10**). These 3 positive brains were having a mild penetration of the dye.

The Repo-RNAi 2 flies have a longer period of paralysis before death than the Repo-RNAi 1 (**Figure 5.4A** and **B**). Moreover, the males start to have motor defects 4 days before the females. Indeed, males have locomotor defects from 8 days while the females show motor defects from 12 days (**Figure 5.4B** right and lower panels). I took advantage of this difference to assess the BBB integrity at 9 days. At this time point, the males just start to have motor defects while the female do not. Out of 11 females, 3 had mild penetration of the dye and the rest had none. Out of 8 males, 3 had strong penetration, 3 were mild and 2 had none. In the control flies, 2 out of 6 for both males and females had mild penetration of the dye. These flies were assessed for climbing assay just prior dye injection on day 8. The females Repo-RNAi 2 showed no difference compared to control. However, 3 males were not climbing at all, 3 were variable between each replicate and 2 had no defects (**Figure 5.4B**, lower graph). Thus, the ratios of impaired motor functions correlate with the ratios of the BBB defects. Another time point was done at 14 days when the female Repo-RNAi 2 flies are paralyzed, just prior to death. In control flies, 3 out of 10 had mild dye penetration while the rest had none. In Repo-RNAi 2 female flies, out of 7, 1 had no penetration, 2 had mild and 4 had strong dye penetration, which is similar to the Repo-RNAi 1 flies at 8 days. Altogether, these data suggest a strong correlation between the strength of the Repo knock-down, the onset of the motor defects and the BBB leakage. Indeed, an increase in BBB defects appears just prior motor impairment and strong leakage of the dye is visible when the flies are paralyzed, just before the death. However, even though there is a strong correlation between the phenotypes, it remains unclear whether the BBB defects are causative responsible of the motor defects and/or reduced lifespan.

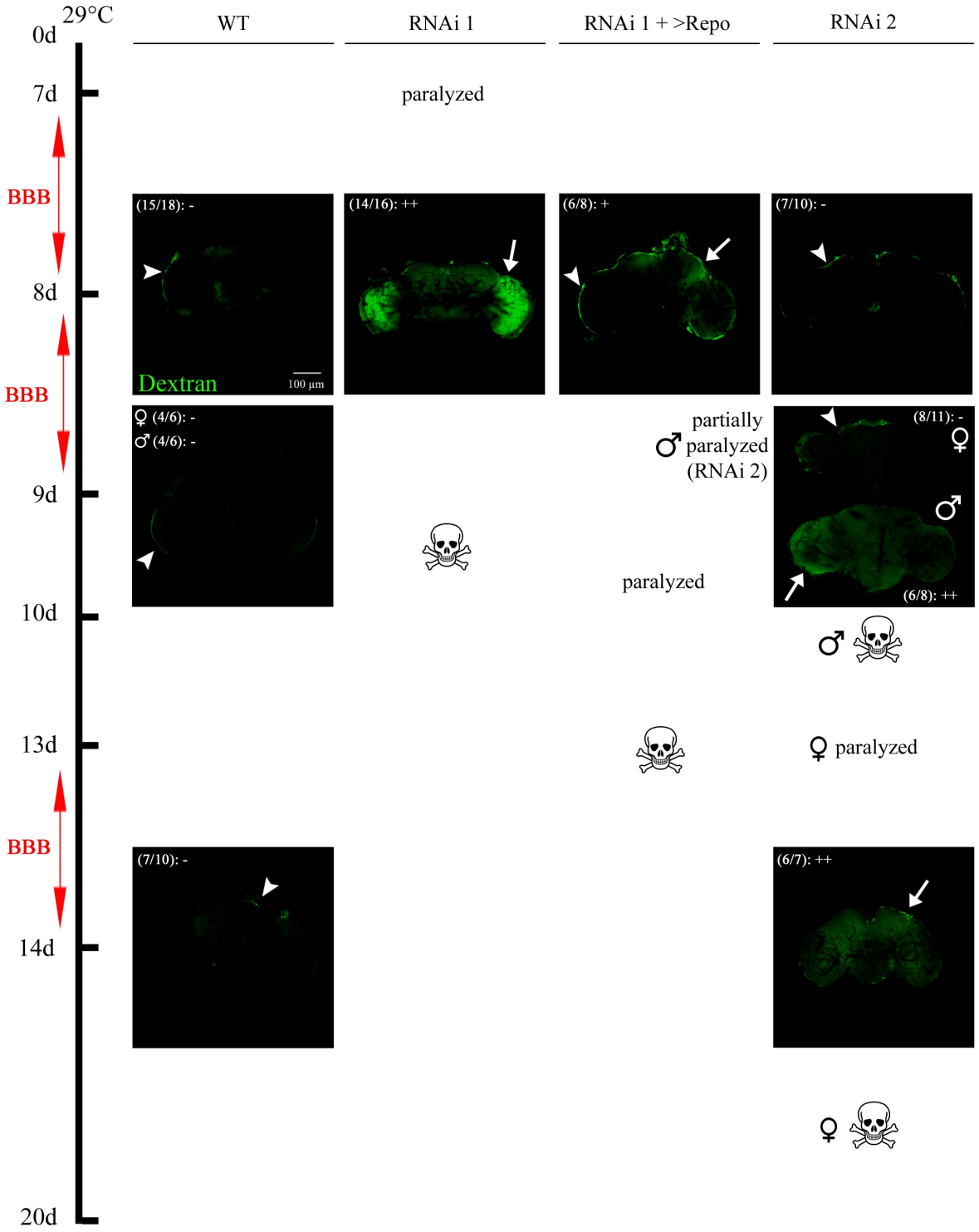


Figure 5.10: *repo* maintains the BBB integrity in adult brain. The genotypes are: *RepoGal4, UbiGal80^{ts}* crossed with either *w^{1118}* as control or the transgenes on top of each column in the figure. The BBB is assessed by the injection of a Dextran dye at the basis of the first pair of legs, after 7, 8 or 13 days at 29°C. The brains are dissected the day after (18-24h). There is a strong accumulation of the dye inside the brain just before the death in both RNAi 1 and 2 conditions. In the rescue experiment, a beginning of infiltration precedes paralysis state. Arrows show sites of infiltration and arrowheads for sites of accumulation of the dye outside the brain. 6 to 10 flies were injected for each conditions. The left bar is not to scale. -: no penetration, +: mild penetration and ++: strong penetration. The right number between the bracket is the total number of brains assessed.

5.2.5.3 Adult *repo* expression acts non-autonomously on the photoreceptor neuron morphology

Glial dysfunction, through probable defects in neurotransmitter recycling and BBB maintenance, will surely affect neurons given that the Repo-RNAi flies present strong motor defects. It is possible that there might be also morphological dysfunctions in the circuitry (beside functional dysfunctions). However, in most cases the really complex intermixing of neuronal network, including axons, dendrites, synapses and glia, makes difficult to visualize altered morphology. The visual system offers the unique opportunity of looking at neuronal morphology, which is organized stereotypically and has neuronal cell bodies physically separated from the glia. In this system, eventual morphological problems can be assessed more confidently and quantified. The photoreceptor neurons are present in the retina and send their axons either into the lamina or medulla of the optic lobe and form synapses with either corresponding lamina or medulla interneurons. However, glial cells are not present in the retina and sit only at the base of it and surround the lamina and medulla neuropils while ensheathing the respective neurons (**Figure 5.11B**, Optic lobe panels). Lamina and medulla astrocyte-like glia, also called epithelial glia, send their projections toward the synaptic contact between the photoreceptor neurons and the interneurons of the optic lobes (Morante et al. 2004). Glial cells are known to uptake histamine (neurotransmitter of these neurons) from the synapse and recycle it in cooperation with supportive cells of the retina back to the photoreceptor neurons (Borycz et al. 2002, Chaturvedi et al. 2014). Thus, *Drosophila* retina provides a good in vivo model for studying non-autonomous effect from glia to neurons.

The retina is composed of roughly 800 ommatidia, each of these subunits containing 8 photoreceptors surrounded by supportive cells such as pigment cells, cone cells or bristle. The photoreceptor neurons 7 and 8 are on top of each other and as a result, in each retina cross-section, only 7 are visible. Each photoreceptor possesses one rhabdomere, which is the folding of the plasma membrane at the apical part of the ommatidium. The pattern of retina sections is a series of perfectly aligned repetitive motifs (hexagon) composed of 7

black dots (rhabdomeres, membrane-rich structure). This repetitive structure can be easily observed by doing tangential sections of fly retina embedded in resin and stained with osmium.

Even though it has been confirmed that the RNAi is not expressed during development and does not downregulate *repo*, the structure of the retina of Repo-RNAi flies kept at 18°C was still evaluated. As expected, no difference with wild-type retina could be observed. The 7 photoreceptors visible were present in all ommatidia and the overall structure of the photoreceptors and retina was not affected. Then, the Repo-RNAi flies were aged 7 days at 29°C alongside control flies, which have the same driver but not expressing the RNAi. Interestingly, no sign of cell degeneration was observed as all the photoreceptors were present in all ommatidia. However, at the top of the retina, where the cell bodies of the photoreceptor neurons and pigment cells sit, the pigment cells surrounding the photoreceptor neurons seem swallowed (black arrowheads in **Figure 5.11A**, localized on the schematic), potentially compressing the neuronal cell bodies. Surprisingly, in some cases, 13 to 15 rhabdomeres could be seen within one ommatidium, even though there were still 7 neuronal cell bodies (black arrows in **Figure 5.11A** and insets for the Repo-RNAi 29°C condition, represented in the top schematic). This result shows that there are extra numbers of rhabdomeres, potentially split, in some photoreceptor neurons. This phenotype suggests a substantial reorganization of the actin cytoskeleton and the membrane in order to split or generate new rhabdomeres, which is a complex active folding of the membrane microvillae. This has only been observed in *repo* hypomorph mutants in which the mutation was present through developmental stages. In the rare cases shown of split/new rhabdomeres, there was one extra per ommatidia and often accompanied by degeneration of the neighbouring ommatidia (Xiong et al. 1995). In the case of adult specific knock-down, there is no neuronal degeneration and sometimes doubled the number of expected rhabdomeres. The interpretation of this phenotype is rather complicated as it has only been reported once, 20 years ago, without deeper understanding.

In order to understand better the morphological defects observed in the tangential sections, the entire visual system morphology was investigated by doing horizontal sections of the

eye. The focus was on four different stereotypical regions, the retina, with long aligned tubular structures, the lamina and medulla neuropils and the fibrous optic chiasm between both neuropils, which has a typical wide angle made by the split of neuronal tracts reaching each side of the medulla. Also, it is noteworthy that there are only 2-3 sections per eye where the photoreceptor neurons are seen aligned in one section from apical to basal part. Thus, it increases the confidence of the specificity of the observed area between the samples. Confirming the previous results of the tangential sections, the pigment cells seemed either swallowed or compacted. In both type of controls, these supportive cells (SC) form spike-like structure on the apical side. However, after 7 days at 29°C, the Repo-RNAi retina do not have clear spike-like structures (**Figure 5.11B**). Also, instead of long and aligned tubular structures present in both controls, the photoreceptor neurons in the RNAi condition were curved and appeared shorter. It is also possible that the increase in volume of the supportive cells exert a mechanical pressure onto the neurons resulting in the folding. However, the weakness of the tissue due to a potential lack of mechanical support resulting in the collapse of the structure during the embedding of the heads cannot be ruled out. Thus, it is difficult to conclude on an overall reduction of the size of either the retina or the photoreceptor neurons.

Then, both lamina and medulla neuropil sizes were analysed since the photoreceptor neurons 1-6 and 7-8 send their axons in their corresponding targeted neuropils to make synaptic connections with lamina and medulla neurons respectively. The size of the medulla does not change in RNAi flies (data not shown) even though it appears more distant from the edge of the optic lobes, revealing more cell bodies in between. Furthermore, between the lamina and the medulla, the optic chiasm wide angle made by the fibres from photoreceptor neurons 7 and 8 and the lamina neurons seems less open, indicating a change in the organization of cells and fibres in the optic lobe (**Figure 5.11B**, Optic lobe panels). The size of the lamina was also evaluated. Contrary to the medulla, the lamina was significantly reduced by 26% in width (**Figure 5.11B**, graph) potentially due to loss of fibres. Another visible aligned structure in the controls was white fibres coming from the optic chiasm entering the medulla neuropil. In both controls about 30 to 32 of them were visible (**Figure 5.11B**, Optic lobe panel). However, these white fibrous

structures were absent in the Repo-RNAi flies and some black ones were visible instead. The length from the entrance of the medulla to the tip of these structures, white and black ones, was similar (data not shown), suggesting that these fibres target a similar layer in the medulla. These white structures in the medulla resemble closely the axons of the lamina neurons 1 to 3 (LN in the **Figure 5.11B**), which target the medulla layers M1, M2 and M5 (Pantazis et al. 2008). However, this hypothesis would need to be confirmed by using a specific Gal4 driver for these neurons as the medulla is reached by membrane tracts from photoreceptor neurons, lamina and medulla neurons but also epithelial glial cells. Furthermore, there are only 15 to 20 black structures visible instead of the 30-32 white ones. It is thus not possible to confirm using these pictures whether the white and black structures are of the same origin and degenerate or if the black ones are different and become visible because of the loss of the white ones. Taken together, these data demonstrate that the downregulation of *repo* during only 7 days is enough to the trigger of substantial morphological changes in both the retina, where *repo* + cells are absent. The folding of the photoreceptor neurons and the morphological changes of the pigment cells and in the optic lobe suggest dramatic morphological changes of the neuronal tracts in the nervous system.

Since there was an abnormal morphology of the photoreceptor neuron cell bodies, their connectivity has also been investigated. Using a specific reporter expressed specifically in the entire photoreceptor 7 including the axon, the morphology and the synaptic region of the axons in the medulla was assessed. The synaptic region of the photoreceptor 7 in the medulla is well characterized and its aligned structure can be easily evaluated. The alignment of the synaptic regions of the photoreceptor neurons 7 is conserved in the Repo-RNAi flies as it is seen by confocal imaging using the *Rh4-LacZ* reporter with or without Repo-RNAi 1 expressed (**Figure 5.11B**, lower panels). While the alignment of the synapses between the control and Repo-RNAi conditions were similar, reaching the M6 layer of the medulla, the number of axons in the Repo-RNAi condition was difficult to assess because of the variability between the samples. Indeed, as observed in **Figure 5.11B**, there are often dark areas hiding potential tracts. However, out of 12 brains imaged for each genotype, the intensity of the signal and the number of axons were reduced in most cases (**Figure 5.12**).

A better staining using for instance the antibody 24B10 (staining the photoreceptor 7 and 8) would possibly allow a more accurate evaluation of a possible loss of photoreceptor neuron axons.

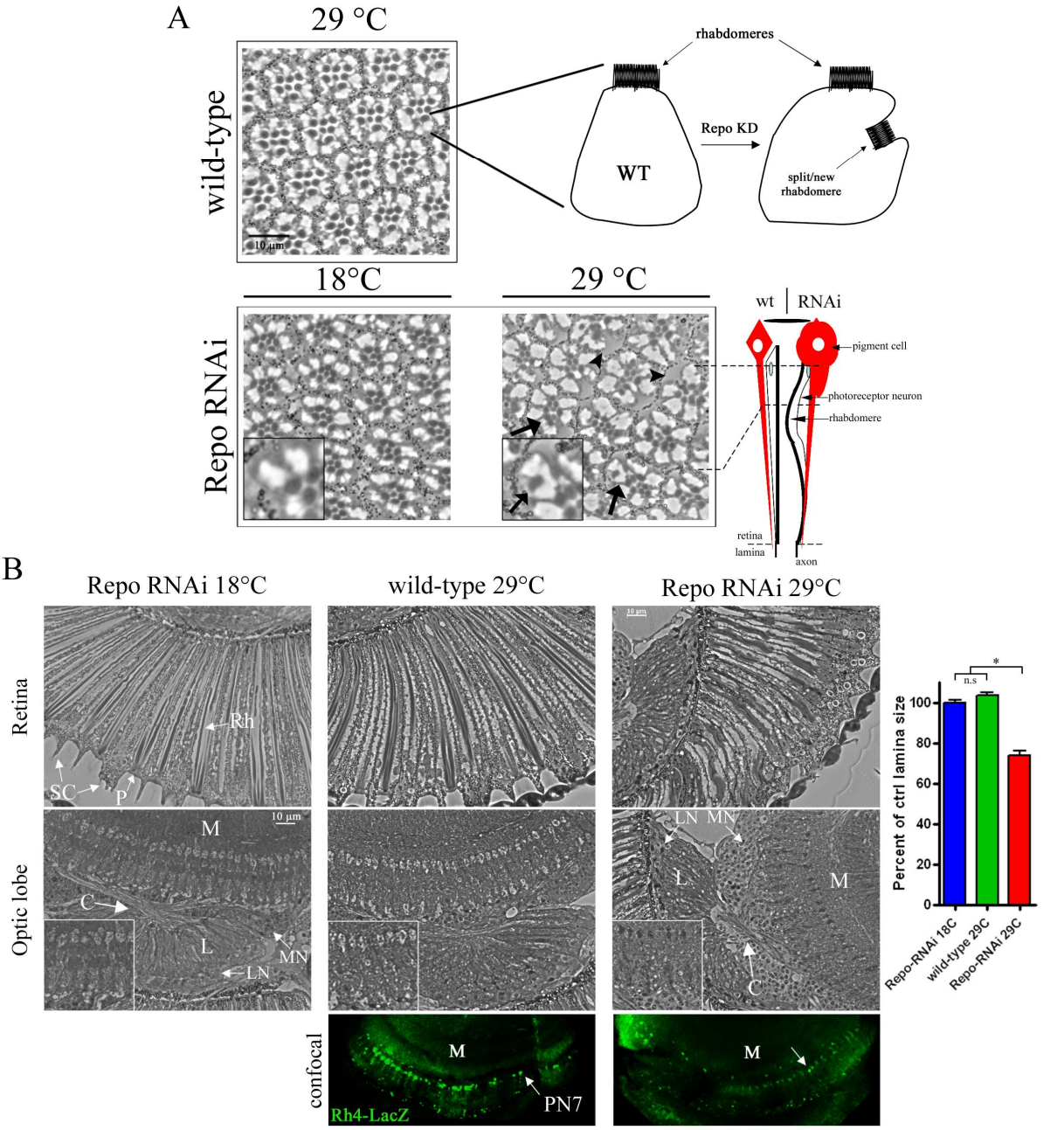


Figure 5.11: *repo* KD non-autonomously affects photoreceptor neurons morphology. All genotypes are: *Repo-Gal4, UbiGal80^{ts}xUAS-transgenes.* w1118 is used as control. **(A)** Retina tangential sections of control flies or Repo RNAi 1 in all glial cells. The RNAi expression is only activated at 29°C, and the sections are made after 7 days at 29°C, when the flies stop moving and about to die. The section at 18°C controls for the block of the RNAi expression during development. The arrowheads show swallowed supportive cells and normal arrows indicate photoreceptor neurons with extra numbers of rhabdomeres. The schematic next to the pictures are a representation of the morphological changes of the pigment cells and photoreceptor neurons in the retina. **(B)** Retina and optic lobe horizontal sections of the different conditions, similar to (A). The photoreceptor neurons of the Repo RNAi 1 29°C are curved and the retina seems thinner. The spikes of the suportive cells are flatten or swallowed. In the medulla, the aligned white structures are lost and a black one is visible instead. It is also to notice that the angle of the optic chiasm fibers is less open, revealing the presence of more cells between the lamina and the medulla. Rh:Rhabdomere, SC:Supportive Cells, P:Photoreceptor neurons, C:Optic Chiasm, M:Medulla, MN:Medulla neurons, L:Lamina, LN:Lamina Neurons. The lower panels represent the immunostaining anti-ß-Gal in green of the photoreceptor neurons 7 (PN7) reaching the layer M6 of the medulla. The graph next to the horizontal scetions represent the quantification of the width of the lamina. 3 lamina per genotype were assessed. The error bars are SEM. *:p<0.05. One way ANOVA with Dunn's multiple comparison test was done for statistical analysis.

The glial cells being present only in the optic lobe and not in the retina itself, the changes of the cellular morphology for both pigment cells and photoreceptor neurons happen in an indirect way, potentially through dysfunctions in the downstream interactions between glia and neurons or glia and supportive cells. The two main interactions between glia and photoreceptor neurons are at the synaptic level and for the insulation of their axons once entering into the optic lobe. Also, as mentioned in the study by Chaturvedi et al. (Chaturvedi et al. 2014), the glial cells highly communicate with support cells of the retina. It is thus possible that the disrupted morphology of the pigment cells comes from an abnormal communication between these two cell types.

Following the observations that there is a loss of membrane tracts in the medulla or at least a dramatic disorganization of the medulla at the synaptic layer, the integrity of the axonal bundles in the brain was assessed. Indeed, it has been shown that killing most of the glial cells specifically at the adult stage was resulting in some specific bundles degeneration called secondary axonal tracts (SATs), depending on their interactions with glial cells (Spindler et al. 2009). However, even though most of the glial cells were dead, very few of these SATs were affected by a degenerative process. In order to see if similar axonal tracts degeneration was happening when *repo* was downregulated, brains of 7 days old flies at 29°C were immunostained with Neuroglian, the same marker used in the above mentioned study for these SATs. However, no degenerated SATs could be observed and there were no obvious differences between the wild-type and Repo-RNAi flies (**Figure 5.12**). It is actually not surprising since the phenotype when most of the glial cells were dead was very mild. It means that the fasciculation of these tracts is rather stable even when the glial cells are dramatically disturbed. Moreover, the optic chiasm between the lamina and medulla neuropils is still present and does not seem to be degenerating, supporting the Neuroglian staining.

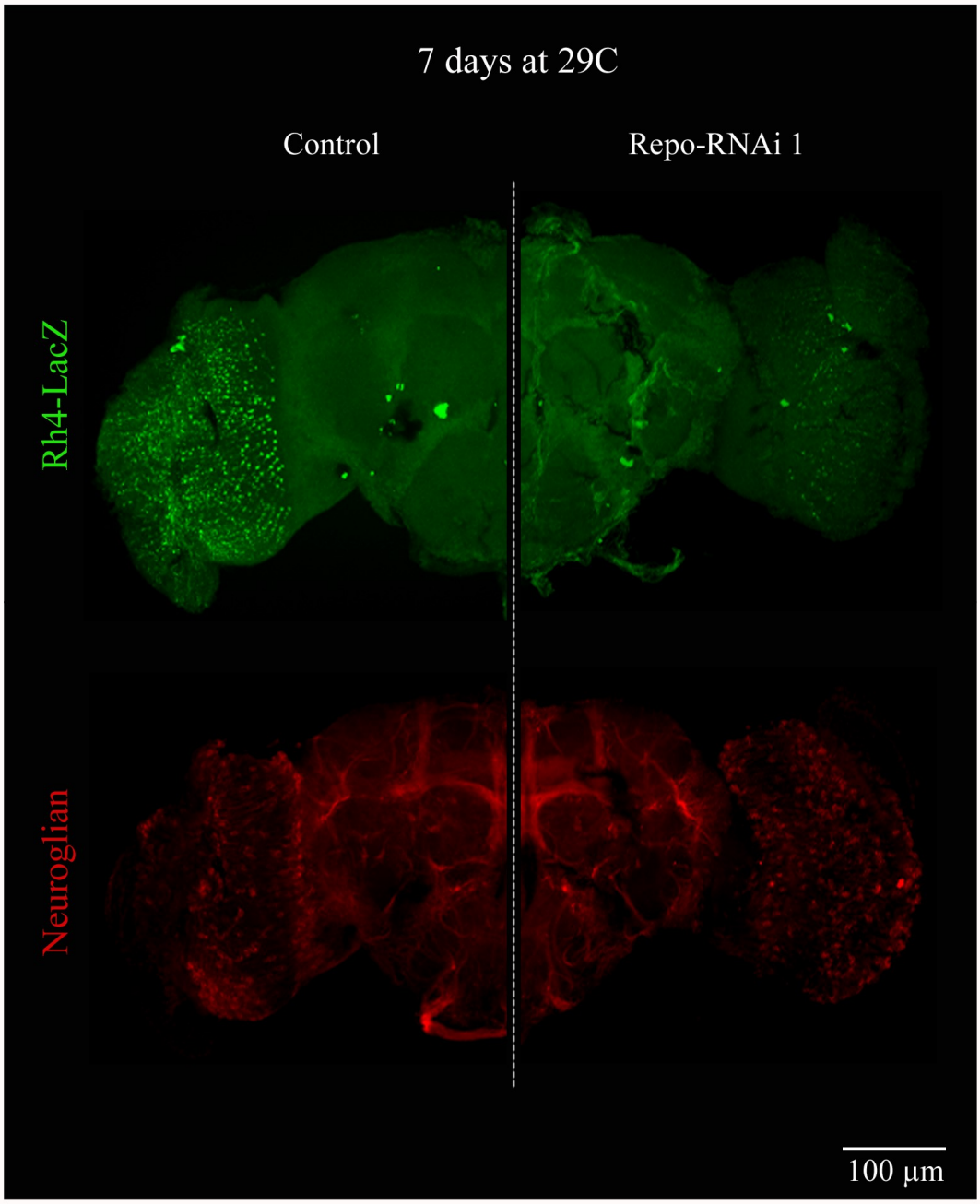


Figure 5.12: Immunostaining of the photoreceptor neurons R7 and secondary axonal tracts. The genotypes are as follow: *Repo-Gal4, TubGal80^{ts}; Rh4-LacZ* crossed either with *w^{1118}* as control or *UAS-Repo-RNAi 1.* Anti-ß-Gal and Neuroglian antibodies in green and red respectively were used on adult brains. The pictures represent Z-projections of half brains imaged by confocal microscopy.

5.3 Discussion

5.3.1 *miR-1* is involved in mesoderm-neuroectoderm fate determination during embryogenesis

So far, *miR-1* has been only studied in mesodermal fate commitment and muscle cell differentiation. In mammalian cells, Izarra et al. (Izarra et al. 2014) show that the expression of *miR-1* triggers mesodermal fate in embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), partly through the downregulation of neural fate factors. However, *in vivo*, in *Drosophila*, *miR-1 KO* (-/-) larvae appear normal in starved conditions that keep them into early larval stage (L1) but die when fed, which normally triggers muscle cell to enter endocycle in order to grow fast without division. However, the *miR-1 KO* phenotype does not phenocopy entirely the phenotypes of genes involved in the endocycle. Thus, its function in muscle integrity and growth remains unclear. The main hypothesis is that *miR-1* would maintain muscle cells identity by downregulating promiscuously transcribed non-muscle genes. These "ectopic" transcriptions could be the result of exceedingly active nuclei in muscle undergoing rapid growth. Also, before being expressed in myogenic cells, likely through the activation by the transcription factor *Mef2*, *miR-1* is expressed in the entire mesoderm, including presumptive immune cells, through its activation by *twist*. Similarly to what was observed in mammalian stem cells, this early expression could indicate a role in cell fate commitment, to ensure that the cells will differentiate toward the desired fate. The results provided in this thesis, combined to the *twist KO* (-/-) from our collaborators support this theory. The redundancy of transcription factor expressions in different tissue would explain the need for safeguards to prevent ectopic expression of unspecific genes. The expression of *gcm*, first transiently in the mesoderm and then in the neuroectoderm, supposedly activating different sets of transcription factors, is the perfect example. Moreover, *gcm* has been more recently found to be involved in post-embryonic haematopoiesis by interacting with *dPIAS* and acting upstream the *JAK/STAT* pathway (Jacques et al. 2009). In this case, it is possible that the expression of *gcm* in non-neuroectodermal cells could potentially activate the ectopic

transcription of *repo*. Being a transcription factor activating multiple glia specific functions, a way to inhibit such potential ectopic expression would be to express specific miRNAs, which have a broad spectrum that allows them to downregulate several genes at the same time in a tissue specific manner.

Also, the fact that *twist KO* and *miR-1 KO* embryos only display a weak phenotype, with around 10 cells in each case positive for Repo and Srp, shows that there are other mechanisms partly redundant to protect against such change of fate. However, whether those cells would become glial cells is still not clear as it would require the activation of other genes, which might be downregulated by other miRNAs or inactivated by other factors, such as *Srp* itself.

The ectopic expression of *twist* in the neuroectoderm decreased dramatically its organisation and the number of glial cells (data not shown). Even though I could not reproduce a similar result by the overexpression of *miR-1* at this stage of embryogenesis, it would be interesting to know whether the *twist* phenotype is at least partly due to the activation of *miR-1* by repeating the *twist* overexpression in a *miR-1 KO* background. The larval brain phenotype obtained by the ectopic expression of *miR-1* indicates a role of targeted genes in glia division and/or migration. The impairment of the glia localization in the presumptive optic lobe in L3 larval stage is actually very similar to the one of *repo* mutant, where it was demonstrated that *repo* was involved in glial migration (Xiong et al. 1994). *repo* is a direct target of *miR-1*, being upregulated in the mesoderm in *miR-1 KO* embryos and downregulated by the ectopic expression of *miR-1* in adult glia and in S2 cells. However, the failure to rescue the phenotypes in L3 larval brain and partially in the adult with the overexpression of a miRNAs insensitive *repo* transgene (lacking its 3'UTR) indicates that *miR-1* might downregulate other important genes in the glia. *Pros* could be such a candidate gene as it has been predicted to be downregulated by *miR-1*. Indeed, *Pros* is involved in glial cell division during development and its downregulation in larvae could lead to brain growth defects, by affecting the insulin pathway for instance. However, it is highly possible that the defects observed are the results of a combination of several genes downregulated whereby the overexpression of only one would not be enough to counteract

the effects of a miRNA. Indeed, the downregulation of *inx1* and especially *inx2* in surface glia led to a smaller brain in L3 larvae and thinner and elongated VNC in the case of *inx2*, very similar to what is observed with *miR-1* overexpression in larvae (Spéder et al. 2014). Interestingly, *inx2* is predicted by one miRNA targets website (microRNA.org) to be targeted by *miR-1*. This reduction in larval brain size is due to the blockage of NSCs into quiescence. The re-activation of quiescent NSCs is nutrient dependent and requires subperineurial glial gap junction, formed by the innexins family. Thus, if such gap junctions or related proteins in similar pathways are downregulated by *miR-1*, it would lead to a very similar phenotype.

In summary, *miR-1* seems to be an active player in cell fate commitment during development, both in invertebrates and vertebrates. Its role has been extensively studied in muscle and cardiac cells in different organisms, but it has also been reported as being a candidate tumour-suppressor (Hudson et al. 2012). In this thesis, I add to *miR-1* another potential regulatory role, which would happen earlier in development, during early embryogenesis, where it would participate to the correct differentiation of hemocytes in the mesoderm, by inhibiting the glial gene *repo*.

5.3.2 *repo* expression in adult glia is essential for *Drosophila* CNS homeostasis

repo expression starts during the embryogenesis, triggered by *gcm*, but remains constantly expressed until adulthood. However, depending on the developmental stage, its function might be different. Indeed, its role in glial migration and terminal differentiation is not needed in adult differentiated cells. From embryogenesis, *repo* activates glia specific genes such as *dEAAT1*/*2* and *Gs2*, or glial *wg* in the larvae. This function of transcriptional activator seems to remain in adult *Drosophila*, suggested by the fact that *repo* is needed to maintain the transcription of several glial-specific genes. It is actually one of the few cases in which an important developmental transcription factor keeps controlling the transcription of some of the same developmental target genes in the adult. It is possible that its specific developmental functions require other factors that are not present in adult glial cells, such

as *ttk69* or *gcm*. It has been demonstrated that during embryogenesis, depending on the type of glial cells, *repo* cooperates with different factors to activate specific subsets of genes, which is the case for *loco* for instance in the BBB glia. Glial cells have different specific functions in the adult as well, even though *repo* is expressed in all of them. It suggests that this specificity might still be needed in order to maintain specific gene transcription. *Gat* is expressed only in astrocyte-like glia while *loco* is supposed to be subperineurial glia specific. Thus, a complex regulatory pathway, implicating *repo*, may regulate the expression of specific glial genes in specific subsets glial cells as it is the case during development with *ttk69* or *ptnp1*.

To add another layer of complexity to the study of *repo* function in the adult fly, the regulation of *nGFP* expression downstream the *repo* promoter suggests that it participates to the regulation of its own transcriptional activity, by a complex feedback loop. However, the *repo* promoter used to generate the *Repo-nGFP* reporter is only the sequence directly upstream the coding region, which might lack some other regulatory sequences. A potential feedback loop is however further supported by the fact that a high Repo protein level seems to be needed in order to maintain its own transcription and suggest a potential regulation as a transcriptional bistable switch. Even though the RNAi presence/absence after 7 and 20 days at 18°C has to be checked, it is very unlikely that a pool of small RNA is stable and active that long within a cell, downregulating newly produced mRNA for 20 days. It is thus likely that a small amount of Repo protein is able to maintain a low level of transcription, enough to delay the death by more than 20 days. It would be interesting to investigate the molecular mechanisms behind this regulation. One possibility for the presence of this residual amount when *repo* is overexpressed beside its RNAi is that the inactivation of the gene is mosaic, meaning that some cells are more affected than others. A staining of the brains in this condition would allow us to see whether all the cells have low levels or if some have higher levels than others. This hypothesis comes from the staining of the full *repo* KD brains (using RNAi 1), where it is sometimes possible to see very small group of sparse glial cells, or single isolated cells, positive for Repo, though to a low level (data not shown), again suggesting the possibility of a transcription bistable switch regulation of

repo. These very few cells expressing low levels of *repo* do not allow detection by western blot as seen previously in the Result section (**5.2**).

These results bring on one important question. How can the promoter be switched off irreversibly? It is possible that the *repo* promoter is also regulated by some epigenetic factors. The presence of Repo protein could be necessary to maintain the chromatin open and allow the transcription of the gene. However, when the protein is lost or down to a very low level, the chromatin could be modified and prevent the access to the promoter region to new transcription factors. In this case, newly formed Repo protein would not be able to reactivate its own promoter. To verify this hypothesis, we would need to be able to downregulate fully *repo* first, and then activate a transient exogenous expression of *repo* to see if endogenous Repo protein is formed. However, with the tools available, it is currently not possible to answer this question. Moreover, the *Repo-nGFP* reporter seems to be also affected by this auto-regulation. It suggests that this potential epigenetic mechanism is chromosome position independent and might be encoded in the 4.2kb promoter region, upstream the coding sequence. It would be interesting to see whether the nGFP level remains high in the rescue setting, when the RNAi 1 is co-expressed with the *UAS-Repo* line. It could help understand whether this residual amount of Repo protein is able to maintain the transcription of *nGFP* through the activation of the 4.2kb *repo* promoter.

This complex regulation brings the question of the efficiency of the *Repo-Gal4* driver in order to carry out rescue experiments as done here. Indeed, *Repo-Gal4* driver is a knock-in, meaning that the Gal4 expression is dependent on the activation of the endogenous *repo* promoter. This could explain the failure to rescue the lifespan phenotypes because the Gal4 protein levels may be too low to activate a high expression of the desired transgenes. In order to overcome this problem, it would help to generate a line that would activate an *Actin-Gal4* driver specifically in glial cells using the FLP/FRT method. This line would allow the transgene expression independently from the presence of Repo protein, unlike potentially the *Repo-Gal4* driver.

It has also been demonstrated that *repo* is necessary to maintain certain transcription levels of specific glial genes. In the group of genes evaluated for a potential *repo*-dependence, at least four of them seem to have their transcription affected. Three are involved in neurotransmitter clearance (*dEAAT1* and *Gat*) and recycling (*Gs2*) and one in blood brain barrier maintenance (*loco*). However, only Gat protein levels have been confirmed to be also downregulated as we do not have antibodies for the others. Neurotransmitter clearance and recycling is a very important process to maintain a good synaptic activity and avoid neurotransmitter excess in the cleft, which triggers toxicity. Also, if the neurotransmitters are recycled back to the neurons, the pool available for the neurons to fire will decrease and thus affect the capacity for the neurons to activate properly the post-synaptic neurons. Glial cells are known to not only clear up the synaptic cleft but also regulate the synaptic activity through different receptors that allow them to compete with receptors from the post-synaptic neurons. It has already been demonstrated that the downregulation of some of the neurotransmitter receptors, such as *dEAAT1* and *Gat*, affects motility, a phenotype observed when *repo* is downregulated. However, the downregulation of *Gs2* does not alter the motility and is not necessary to support the basic functional properties of the Dorso-Longitudinal-Muscle (DLM) neuromuscular synapses (McCabe et al. 2015). However, it is possible that its activity becomes necessary to sustain glutamate release during prolonged periods of synaptic activity. The alteration of the transcription of such genes, may be among others that have not been assessed in the qPCR, will certainly affect this major function of glial cells, mostly astrocyte-like glia. Indeed, this cell type sits at the border of the neuropils where all the synapses are, and send their projections toward them. Even though it has not been shown yet that the projections are in very close vicinity with the synapse itself, contrary to what has already been observed in mammals, the fact that these cells send membrane projections in synaptic rich regions, and that they are specifically expressing neurotransmitter transporters, highly suggest a similar role to the mammalian astrocytes.

loco on its side is known to be involved in the *Drosophila* "BBB" maintenance through a GPCR signalling pathway. This regulation would be important for the maintenance of a dynamic actin structures to keep the membranes gap sealed by septate junctions (Hatan et

al. 2011). The deregulation of this signalling pathway could disrupt the proper sealing between the subperineurial glial cells, forming the tight BBB layer. Acting in a complex with different G proteins, an overexpression or downregulation of *loco* would have both detrimental effects on its function. Thus, the decrease in the transcriptional activity of the *loco* promoter, leading to lower *loco* mRNA levels, could affect this signalling pathway and disrupt, at least partially, the tightness of the septate junctions formed between the subperineurial glia. It has been shown that a Dextran dye injected in the thorax of the fly could penetrate the brain when *repo* was knocked-down. It suggests that the "BBB" function of certain glial cell types is disrupted and possibly does not maintain the brain in a tightly regulated ion environment due to the BBB leakage. This specific extra-cellular ion environment is necessary to maintain healthy neuronal activity, by keeping a good ratio of extracellular ions to allow efficient axon potentials. Indeed, the hemolymph, present all around the CNS, has a very high potassium concentration (~26mM) (Limmer et al. 2014). While the CNS extracellular K+ concentration is known to be at least around 10 times lower than that, a leakage of such ion from the hemolymph would dramatically affect neuronal function (Limmer et al. 2014). As suggested by the co-overexpression of loco beside the RNAi 1, which decreased even further the lifespan, it is possible that subperineurial glia dysfunction can participate to the phenotype by disrupting septate junctions at the BBB and thus modifying the ion environment leading to neuronal defects.

As I have shown in the results and explained above, the *repo*-activated genes seem to be involved in many different functions. Indeed, its downregulation triggers a lot of different defects such as BBB impairment, motor defects and changes in neuronal morphology and possibly connectivity. We yet have to investigate the neuronal activity in Repo KD condition, but with the strong decrease of glial GABA transporter, expressed at the synaptic level and the BBB leakage that will likely trigger massive changes in the extracellular environment, it is highly possible that the neuronal activity is impaired. Also, according to my results, this impairment may be rather general to all neurons as rescuing *repo* expression in the VNC (and potentially in the PNS depending to the *Tsh-Gal80* expression pattern outside the CNS) did not improve the locomotor phenotype. However, the *repo* expression level in the VNC would need to be assessed in order to know whether *Tsh-*

Gal80 fully rescues the expression or only partially. Indeed, I have demonstrated that a tight regulation of Repo protein level is necessary to maintain glial functions. Furthermore, the photoreceptor neurons, which are in close contact with glia at their synapse, are also dramatically affected. This may cause blindness, however, it is unlikely that this potential blindness triggers motor defects as "blind" flies have only very mild motor behaviour defects and live perfectly well, the eyes being not necessary for the fly to live in laboratory conditions. However, if *Tsh-Gal80* is not expressed in sensory organs such as the chordotonal organs, it is possible that defects in the sensory organs could result in locomotor deficit similar to what observed in this study. To verify this hypothesis, *repo* would need to be downregulated specifically in the glial cells in those sensory organs to assess the locomotor activity and also potentially the lifespan.

repo is expressed in all the glial cells in the entire CNS and PNS so it is unlikely that only a specific neuronal population is affected by general glial impairment. Surprisingly, the use of more specific drivers to target *repo* downregulation to subtypes of glial cells did not have any effects, apart from the use of *dEAAT1-Gal4* (mild lifespan decrease, which is a combination of several glial subtypes, mostly astrocytes, cortex glia and ensheathing glia). This strengthens the hypothesis that such a strong lethality comes from an addition of defects from different subtypes of glia and not one dominant population involved, such as the astrocyte-like glia in the case of polyQ Atro toxicity. Also, the strength of the Gal4 drivers used can be an issue as it is possible that *Alrm-Gal4* is weaker than *dEAAT1-Gal4* in astrocyte-like glia. Moreover, when *Moody-Gal4* driver was used to downregulate *repo* in subperineurial glia (BBB glia), no BBB defect could be observed, questioning the strength of this driver (data not shown). This could explain why the lifespan was not affected in this condition. The use of *Gliotactin-Gal4*, stronger than *Moody-Gal4* for *polyQ-Atro*, could help to understand the importance of BBB breakage if it would reduce the lifespan or trigger dye penetration inside the brain. So far, the reason of the fly death and motor behaviour defects is yet to be fully understood. Indeed, the overexpression of the potential *repo*-downregulated genes did not rescue the phenotypes. However, it is possible that several of these genes have to be co-overexpressed in order to get a rescue in the lifespan since the strong phenotype in such a complex functional assay might come from a

combination of events. A solution may be to look at the rescue of more specific defects at cellular level. For instance, the BBB defects have to be investigated further in order to understand whether it actively participates to the lifespan and locomotion phenotypes since so far we can only correlate the strength of *repo* downregulation to the strength of the BBB leakage. The generation of a Gliotactin-Gal80 line to block the expression of the UAS-Repo-RNAi specifically in BBB glial cells would provide a better understanding on the direct role BBB defects could have in motor defects and/or lethality of *repo* downregulation. However, these results show clearly that the active transcription of *repo*-dependent genes is important to maintain a functional BBB.

The dramatic morphological changes observed in the visual system are also very interesting as a paradigm for non-autonomous effects of glia, especially since they are not in direct contact with the photoreceptor neuron cell bodies. We know that some of the glial neurotransmitter transporters are downregulated but the histamine transporter(s) expressed by the epithelial glial cells surrounding the lamina and medulla has not been investigated. However, the identity of such glial transporters is unclear. Indeed, the staining for the glial specific histamine transporter was present only in the lamina but not in the medulla (Pantazis et al. 2008) and another study has shown its expression in clock neurons (Hong et al. 2006). It highlights the lack of knowledge and characterization of adult glial function in *Drosophila*. The split of rhabdomeres has been reported in a *repo* mutant, which was defective from developmental stages. It is then the first time that such a phenotype is observed specifically in adult. The flies are born with normal photoreceptor neurons and a normal glia organisation in the optic lobes, contrary to the former study, but within 7 days they develop extra rhabdomeres. The fact that the supportive cells are also affected could suggest that there are also defects in the interaction between the glia and those cells. Indeed, a tight cooperation is needed between these two cell types in order to recycle correctly the histamine back to the neuronal cell body. A defect specifically at this level would trigger photoreceptor neuron dysfunction, without the need of impairment at the synaptic level, especially in a system that requires such a high demand in histamine, being constantly secreting the neurotransmitter. However, the different studies investigating the recycling of histamine have not mentioned any abnormalities about extra rhabdomeres.

Either it has not been looked at or there were no such phenotypes. It is also possible that the extra number of rhabdomeres observed in the ommatidia come from the complex folding of the neuronal membrane observed in the horizontal sections of the retina. However, even though the entire structure looks curved, the extra numbers of rhabdomeres observed per tangential section would require a much more disorganized membrane. To confirm this hypothesis, a 3D reconstruction of the horizontal and tangential sections would be needed, which is technically very complicated. The dissection of the retina followed by phalloidin staining did not allow reasonable observations. Indeed, the phalloidin signal, usually concentrated at the rhabdomeres, was also intense in the supportive cells in the apical side of the retina, hiding potential signal deeper in the tissue (data not shown). Overall, the photoreceptor neuron membranes are completely disorganized and the fibre tracts-rich regions in the optic lobe seem dramatically affected by the loss of specific structures and the shrinkage of the lamina. The observations of a smaller lamina neuropil and changes in tracts morphology in the medulla suggest that *repo* expression in adult controls neuronal integrity and morphology. Since other neurotransmitter transporters are dependent on *repo* expression, it is also possible that the histamine recycling process is *repo*-dependent, at least at certain levels and could trigger neurotoxicity in the lamina and/or medulla. It is thus possible that the activity of all these neurons conveying the information of the visual input is impaired. Being a neuronal system particularly demanding in energy, it is possible that the visual system is more sensitive to glial impairment than others and it remains to be investigated whether other neurons and neuropils in the central brain are affected as well. Moreover, it would be interesting to see whether the astrocytic ramifications in the lamina and medulla are affected or not. A glial membrane staining has been done in Repo-KD condition using *Repo-Gal4>UAS-CD8-GFP* to assess potential glial membrane defects but the staining of astrocyte-like glia membranes was weak in this condition while the other glia populations did not seem to be affected (data not shown).

Chapter VI:

Conclusion and future perspectives

The aim of this thesis was to investigate the different roles that *Drosophila* adult glial cells have in the maintenance of a functional nervous system. In order to tackle different aspects of their interactions with neurons, I used different genetic models. The first one uses the glial expression of a gene, *Atro*, responsible for a rare neurodegenerative disease called DRPLA. This approach allowed the investigation of the potential active role glial cells can have in triggering or in the progression of this disorder. It has been already shown that the expression of this polyQ protein in both glia and neurons was leading to autophagic defects. However, it is still unknown how the impairment of the glial cells only, via autophagic defects and surely other dysfunctions, would trigger neuronal impairment that lead to motor defects and early death.

The second genetic model has been used to understand better the role of adult glial cells in the homeostasis of the brain. Indeed, glial cells interact extensively with neurons but are also the barrier with the outside world, regulating what goes in and what goes out. *Drosophila* has been used to model many neurological disorders but the focus was mostly on the molecular mechanisms happening in neurons that would lead to their own degeneration. The importance of glial cells was mostly disregarded. However, recent studies have highlighted the major direct or indirect contributions of glial cells in neurodegenerative diseases. The genetic tools available for studying *Drosophila* have made this invertebrate organism a first choice in the understanding of basic cellular and molecular mechanisms conserved across species. The nervous system development of the fly has been extensively characterized, sometimes to a single-cell resolution. Indeed, in the embryonic VNC, the equivalent of the spinal cord, the origin, location and migration of neurons and glial cells are well documented. However, when it comes to adult functions, very little is known about glial cells functions. During larval development and pupa metamorphosis, many different glial cells are able to engulf specifically neuronal debris, but what about in the adult? While in mammals, astrocytes have been heavily studied in providing energy metabolites to neurons mostly via the lactate shuttle, very little is known about a similar function in adult *Drosophila*. One study has identified the outermost peripheral glia of the BBB as the cells importing trehalose into the brain. And another

study, though again in larvae, has identified some cortex glia that store lipids and thus could be the one bringing the energy the neurons require to function (Kis et al. 2015). If we want to use *Drosophila* as a more complete genetic model for studying nervous system and its dysfunctions, it requires the understanding of the role of each of the players. Thus, I decided to focus on the role of a transcription factor expressed specifically in all the adult glial cells. Its function has been characterized during embryogenesis but very little is known about its role in adult glia. It is a major terminal differentiation factor, triggering the expression of many different glial genes in different subsets of glial cells. Thus, it makes it an ideal candidate to study the adult function of glia in *Drosophila*.

6.1 Different glial cell populations are involved in *Drosophila* DRPLA neuronal impairment

In order to characterize the role of glial cells in the impairment of the nervous system, I first wanted to understand if there were any glial subpopulations more vulnerable to the polyQ toxicity than others. Using different specific glial drivers, I highlighted the importance of a specific subset, the astrocyte-like glia. Indeed, the different drivers used, either overlapping or complementary in expression pattern, having the strongest effect, were expressed in this subpopulation. These results demonstrate that the impairment of specific glial cells is enough to deregulate the nervous system homeostasis, leading to the shortening of the lifespan. The astrocytic population in *Drosophila* has not been well characterized so far. It is known that they express the glutamate transporter *dEAAT1* and the GABA transporter *Gat*, both of them overlapping in their neuropil expressions. The GABA transporter expression completely overlaps with the expression pattern of the specific astrocyte-like Gal4 driver *Alrm-Gal4*. However, the glutamate transporter seems to have a broader expression pattern, suggesting the presence of another astrocyte-like glia population in the *Drosophila* brain, possibly the so called “cortex glia”. Indeed, I have shown that the specific cortex glia Gal4 driver, *NP2222-Gal4*, also partially colocalizes with the *dEAAT1 Gal4/LexA* driver. The hypothesis of a new definition of the cortex glia nomenclature

comes from their positioning, in direct contact with both BBB glia and neurons, similarly to mammalian astrocytes. The only function suggested so far about the cortex glia is its potential role in providing energy to neurons, assumed by its location, storage of lipid droplets in larvae and their membrane surrounding the neuronal cell bodies. Thus, potentially expressing some neurotransmitter transporters and providing energy to the neurons via their contact with BBB glia, this glial subtype could be redefined as a subpopulation of astrocyte-like glia. Also, the cortex glia driver leads to a reduction of the lifespan in the same range of *Alrm-Gal4* when expressing the mutated *polyQ Atro*. The generation of a *dEAAT1-LexA* line in this thesis has helped to emphasize the lack of characterization of the glial populations and functions in the adult brain. Although the expression pattern and level of Gal4 lines have to be taken into account, not always fully representative of the real expression pattern of the endogenous promoter, the different stainings of the dEAAT1 protein seem to show a broader spectrum than the GABA transporter *Gat*, expressed only in the neuropil (Rival et al. 2004, Stork et al. 2014). In addition, the staining of the optic lobe neuropils with a glial histamine transporter has shown that it was present in the lamina but not in the medulla, suggesting a diversity in astrocyte-like glia population in both neuropil, especially since both photoreceptor neurons 7 and 8 projecting in the medulla secrete histamine (Pantazis et al. 2008). In any case, the expression of *polyQ Atro* in neurotransmitter-expressing glial cells leads to a reduced lifespan. Thus, it is to be anticipated that the neurostransmitter clearance and/or recycling functions of these cells are possibly affected in this disease model.

The diversity of the glial populations affecting the lifespan when they are impaired shows the broad detrimental effects on cellular functions in different cells provided by the expression of a mutated protein. It is possible that each of these cell types is affected by a similar dysfunction such as autophagy defects, which would lead to a general impairment of the basic cellular functions. Each cell population having a different function in the CNS, a similar impairment in each of them could have a different impact on the homeostasis of the nervous system. However, the large alterations in transcription seen when *polyQ Atro* is expressed could suggest that more specific functions are affected in different subpopulations and may explain why some glial cells are more sensitive than others, as it is

the case for neurons in DRPLA. To investigate this hypothesis, Gal4 drivers with similar levels of expression for each population of glial cells would be required.

Furthermore, we have now demonstrated in the lab that the autophagic defects observed in our *Drosophila* DRPLA model were also present in a DRPLA mouse model. Thus, it is possible that other defects are also conserved between the models. It would be very interesting to know whether the expression of *polyQ Atro* specifically in mammalian astrocytes, using *Gfap* promoter for instance, would trigger similar effects to the ones seen using *Alrm-Gal4* or *dEAAT1-Gal4* drivers. This would allow a better understanding of the role of glial cells in the progression of the disease in a mammalian model. Indeed, the mouse models generated are not specific to one cell type. It is thus complicated to understand which population of neurons and/or glia is first affected and whether there is a propagation of the impairment overtime directly linked to the connectivity of the neurons or due to the sensitivity of each cell type to the toxicity of the mutated protein. The ability to tell the "chicken from the egg" would allow the development of targeted therapeutic strategies toward the starting point of the development of the disease and thus, possibly delay the onset of the symptoms.

6.2 Using *Drosophila* for screening glia-neuron interactions to uncover new mechanisms

The idea behind the screen was to be able to identify the above-mentioned issues in an indirect way. Instead of being dependent and limited by driver strength to identify how specific glial cell impairment leads to neuronal dysfunction, I targeted the neuronal functions to understand what the pathways affected in neurons that modify glia-neuron interactions are. Indeed, the modulation of neuronal gene expressions that enhances or suppresses the lifespan phenotype due to glial toxicity could give indirect answers on what goes wrong in glial cells. However, I have not been able to pinpoint specific glial functions that could be responsible for the impairment of the glia-neuron interactions and further

work is needed to confirm the screen candidates. Then, these impaired interactions would lead us to a potential specific glial population responsible of this type of interactions. Similarly to the large transcriptional alterations observed with the expression of *polyQ Atro*, a broad range of functions seems to be affected by impaired functions in glial cells. I decided to focus on three membrane proteins, *mfas*, *caps* and *trn*, each of them having differential effects in either glia or neurons.

Nothing is known about *mfas* function in *Drosophila*. It has been studied in embryogenesis and is currently being investigated during metamorphosis by the Salecker group. However, the human ortholog *TGF-ß-induced* protein has been linked mostly to tumour processes such as cell migration or angiogenesis (Skonier et al. 1994, Aitkenhead et al. 2002). By binding to the extracellular matrix components such as collagen, laminin or fibronectin, this transmembrane molecule would regulate many processes in a *TGF-ß*-dependent manner (Irigoyen et al. 2008). *TGF-ß* is known to be an important player in neuroinflammation by modulating astrocytic scar formation (Cekanaviciute et al. 2014) or attenuating microglia activation (Norden et al. 2014) but is also involved directly in neuronal remodelling (Awasaki et al. 2011). It is thus possible that a similar signalling pathway in *Drosophila* regulates glial migration or "activation" by the expression of *mfas* in glia. It is also possible that *mfas* could modulate neuronal and/or glial shape via its binding to the extracellular matrix in a *TGF-ß*-dependent manner.

caps and *trn* have different orthologs. Both of them belong to the family of the leucine rich repeat motif family. *caps* is related to *Lrrn2* and *Lingo-2* while *trn* is related to *Lrrn3* and *Lingo3*. *Lingo-2* polymorphisms have been linked to Parkinson's disease (Su et al. 2012) and *Lrrn2* has been shown to be involved in selective targeting of motor neurons (Andreae et al. 2009), which is similar to *caps* function in *Drosophila* antennal lobes (Hong et al. 2009). Also, the *trn* ortholog *Lrrn3* has been linked to autism spectrum disorder (Sousa et al. 2010) and neuroblastoma (Akter et al. 2011). Less is known about *Lingo-3* but another member of the family, *Lingo-1*, has been linked to neurodegeneration by acting on EGFR-PI3K-Akt signalling leading to the reduction of Purkinje cell survival (Zhou et al. 2012). These studies clearly suggest that Leucine rich repeat molecules have distinct roles during

development and adult stage. Moreover, modulating the expression of these genes, orthologs to *caps* and *trn*, could be a potential therapeutic intervention in order to alleviate symptoms of a wide variety of neurological disorders such as Parkinson's disease or autism spectrum disorders. Deciphering the molecular functions of *caps* and *trn* in the *Drosophila* adult nervous system could help to find new strategies to modulate their interactions in disease conditions such as neurodegenerative diseases.

Indeed, membrane molecules are easier to target with drugs, especially when their knock-down triggers a positive effect. It is believed that few days rescue in *Drosophila* would correspond by translation to few months in humans, which is the effect of most drugs on genetic diseases such as neurodegenerative diseases or cancer. A first challenge is to be able to cross the Blood-Brain-Barrier but the increasing understanding of molecular transport happening at the BBB is already helping the design of new approaches to improve the efficiencies of the drug trans-cellular transport. However, getting the drug into the cell to have its effect is another level of complexity, making extracellular targets more appealing for potential treatments. Glia and neurons interact via different pathways, such as cell adhesion or secretion of molecules binding to receptors, but also via exosomes (Frühbeis et al. 2012). Modulating these interactions in disease conditions could help maintaining for longer a functional or partly functional nervous system that would potentially alleviate symptoms of patients.

6.3 Investigating adult glial functions using the glial transcription factor *repo*

In the first part of the thesis, I aimed at understanding if there were some specific subsets of glial cells more important than others in the participation of the phenotypes of our glial DRPLA model. This led to a first conclusion that *Drosophila* astrocyte-like glia play a major role in the toxicity of *polyQ Atro* expression for the organism viability. Since their functions are not well characterized in adult, a first approach was by modifying neurons

while the glia are impaired to help us understand which glial functions are affected. However, this approach is indirect and the results of the screen have shown how difficult it is to reveal non-cell autonomous effects between glia and neurons.

In the last part of the thesis, I changed the approach by directly investigating adult glial functions in *Drosophila*. For that, I targeted a common glial transcription factor, *repo*, known to control glial gene expression during development. It is known as a terminal differentiation factor but its function in adult remains elusive. *repo* also regulates gene expression in larvae, thus it was likely that as a transcription factor still expressed in adult stage, it could still regulate glial function in the adult. The manipulation of *repo* expression specifically in adult would help to uncover new functions of glial cells. By this mean, it could be possible to discover new interactions between glia and neurons happening in the adult brain.

The downregulation of *repo* in adult glia led to a wide range of molecular and cellular defects in both glia and neurons. However, the cause of the death remains elusive. The short lifespan of the Repo-KD flies shows the importance of the network of *repo*-dependent genes in the viability of the entire organism. It is highly likely the death is the result of cumulative effects from different dysfunctions of the nervous system. The infiltration of the dye through the BBB correlates with both the onset of the motor defects and the early death. It is though possible that both events are the results of different defects. The adult knock-down of *Gat* in glia did not lead to obvious motor defects or reduction of the lifespan (at least until 25 days at 29°C, before the breakdown of the incubator, data not shown). The modulation of *repo*-dependent neurotransmitter transporter expression at the synaptic level might not be the cause of the death itself but might participate by synergy to motor defects or other physiological functions such as memory or vision. The recording of neuronal activity in different regions of the brain would help to differentiate the functions affected by the *repo* downregulation and could link some of them to vital functions necessary for the organism viability. The role of glial cells in energy supply in adult *Drosophila* has not been investigated here. This function has been largely under-investigated in adult *Drosophila* and might be responsible for some of the defects, even

though the cortex glia driver did not give a significant lifespan phenotype. The use of specific Gal80 drivers would be complementary to the Gal4 studies and could highlight some effects not seen using specific Gal4 drivers, especially if a synergy between different defective mechanisms is responsible of the strong phenotypes observed.

Another question arose from the importance of a single transcription factor for such a diversity of glial functions. What is the evolutionary advantage of having such an unstable mechanism so important for the nervous system maintenance? *repo* activates different set of genes in different subtypes of glial cells. Similarly to developmental stages, it is possible that it cooperates with different specific transcription factors in each glial subtype. Even though it does not seem that glial cells can transform into neurons in the adult, possibly due to the lack of specific embryonic factors, it is possible that adult glia could change fate depending on the needs, becoming other glial subtypes. The neuronal debris clearance in adult *Drosophila* has not been well investigated. Thus, the "activation" of glia to do so in *Drosophila* might require a change in transcriptional program, partly controlled by *repo* in order to answer local needs due to a trauma for instance. Thus, the *repo* expression level in different glia subtypes could change locally. It would be interesting to investigate whether the level of *repo* expression in all glial cells is similar and remains constant. Another completely different answer to that question would be that evolution has led to very different mode of reproduction in order for the species to survive (r/K selection theory). While higher organisms give birth to few progenies, the parents taking a greater care for them in order to survive, lower ones such as *Drosophila* can generate hundreds of eggs and the ones not viable will die while the rest will survive, betting on the numbers rather than the "quality". The ones that will have *repo* mutations or variability in expression level will die while the viable ones will survive. Evolution is not perfect and has its drawbacks. A relevant example could be the expansion of the CAG triplets within certain genes. The molecular machinery responsible for DNA replication does not reproduce perfectly the length over generation and leads to toxic effects of the translated protein, which can give rise to a wide range of neurological disorders.

Overall, the adult glial functions remain largely understudied and more effort is needed to characterize them and understand their role in the maintenance of a functional nervous system. A deeper characterization and subdivision of glia subtypes would help to understand specificity of their functions and how to modulate them in order to investigate glia-neuron interactions happening in the adult either in healthy or disease conditions. To this end, more genetic tools are needed along with specific molecular characterization to subdivide glial cells further.

References

Aboobaker, A. A., et al. (2005). "Drosophila microRNAs exhibit diverse spatial expression patterns during embryonic development." Proceedings of the National Academy of Sciences **102**(50): 18017-18022.

Agarwal, A., et al. (2014). "Astrocyte Morphology Is Controlled by Neuron-Derived FGF." Neuron **83**(2): 255-257.

Aitkenhead, M., et al. (2002). "Identification of endothelial cell genes expressed in an in vitro model of angiogenesis: induction of ESM-1, (beta)ig-h3, and NrCAM." Microvascular research **63**(2): 159-171.

Akter, J., et al. (2011). "Expression of NLRR3 Orphan Receptor Gene Is Negatively Regulated by MYCN and Miz-1, and Its Downregulation Is Associated with Unfavorable Outcome in Neuroblastoma." Clinical Cancer Research **17**(21): 6681-6692.

Ameres, S. L., et al. (2013). "Diversifying microRNA sequence and function." Nat Rev Mol Cell Biol **14**(8): 475-488.

Andreae, L. C., et al. (2009). "Chick Lrrn2, a novel downstream effector of Hoxb1 and Shh, functions in the selective targeting of rhombomere 4 motor neurons." Neural Development **4**(1): 27.

Arechavala-Gomeza, V., et al. (2014). "Splicing modulation therapy in the treatment of genetic diseases." The application of clinical genetics **7**: 245-252.

Atallah, N., et al. (2014). "Microglia--performers of the 21st century." Rom J Morphol Embryol **55**(3): 745-765.

Auld, V. J., et al. (1995). "Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in Drosophila." Cell **81**(5): 757-767.

Awasaki, T., et al. (2011). "Glia instruct developmental neuronal remodeling through TGF-β signaling." Nature Neuroscience **14**(7): 821-823.

Awasaki, T., et al. (2008). "Organization and Postembryonic Development of Glial Cells in the Adult Central Brain of Drosophila." Journal of Neuroscience **28**(51): 13742-13753.

Awasaki, T., et al. (2011). "New tools for the analysis of glial cell biology in Drosophila." Glia **59**(9): 1377-1386.

Azevedo, F. A. C., et al. (2009). "Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain." The Journal of comparative neurology **513**(5): 532-541.

Baburamani, A. A., et al. (2014). "Microglia toxicity in preterm brain injury." Reprod Toxicol **48**: 106-112.

Bainton, R. J., et al. (2005). "moody Encodes Two GPCRs that Regulate Cocaine Behaviors and Blood-Brain Barrier Permeability in Drosophila." Cell **123**(1): 145-156.

Baron, U., et al. (1997). "Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential." Nucleic Acids Res **25**(14): 2723-2729.

Bartlett, D. W., et al. (2006). "Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging." Nucleic acids research **34**(1): 322-333.

Bayraktar, O. A., et al. (2015). "Astrocyte Development and Heterogeneity." Cold Spring Harbor Perspectives in Biology **7**(1): a020362.

Beckervordersandforth, R. M., et al. (2008). "Subtypes of glial cells in the Drosophila embryonic ventral nerve cord as related to lineage and gene expression." Mechanisms of development **125**(5-6): 542-557.

Bélanger, M., et al. (2011). "Brain Energy Metabolism: Focus on Astrocyte-Neuron Metabolic Cooperation." Cell Metabolism **14**(6): 724-738.

Benarroch, E. E. (2013). "Microglia: Multiple roles in surveillance, circuit shaping, and response to injury." Neurology **81**(12): 1079-1088.

Besson, M. T., et al. (2010). "Increased energy metabolism rescues glia-induced pathology in a Drosophila model of Huntington's disease." Human Molecular Genetics **19**(17): 3372-3382.

Besson, M. T., et al. (2004). "High Affinity Transport of Taurine by the Drosophila Aspartate Transporter dEAAT2." Journal of Biological Chemistry **280**(8): 6621-6626.

Besson, M. T., et al. (2011). "Involvement of the drosophila taurine/aspartate transporter dEAAT2 in selective olfactory and gustatory perceptions." The Journal of comparative neurology **519**(14): 2734-2757.

Besson, M. T., et al. (1999). "Identification and structural characterization of two genes encoding glutamate transporter homologues differently expressed in the nervous system of Drosophila melanogaster." FEBS letters **443**(2): 97-104.

Bhat, S., et al. (2016). "An accelerated miRNA-based screen implicates Atf-3 in Drosophila odorant receptor expression." Sci Rep **6**: 20109.

Bier, E. (2005). "Drosophila, the golden bug, emerges as a tool for human genetics." Nature reviews Genetics **6**(1): 9-23.

Bina, S., et al. (2010). "Transcriptional targets of Drosophila JAK/STAT pathway signalling as effectors of haematopoietic tumour formation." EMBO reports **11**(3): 201-207.

Borycz, J., et al. (2002). "tan and ebony genes regulate a novel pathway for transmitter metabolism at fly photoreceptor terminals." The Journal of neuroscience : the official journal of the Society for Neuroscience **22**(24): 10549-10557.

Bosch, J. A., et al. (2016). "Persistence of RNAi-Mediated Knockdown in Drosophila Complicates Mosaic Analysis yet Enables Highly Sensitive Lineage Tracing." Genetics.

Bribián, A., et al. (2015). "Decoding astrocyte heterogeneity: New tools for clonal analysis." Neuroscience.

Burda, J. E., et al. (2014). "Reactive gliosis and the multicellular response to CNS damage and disease." Neuron **81**(2): 229-248.

Bushong, E. A., et al. (2002). "Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains." J Neurosci **22**(1): 183-192.

Butt, A. M., et al. (2006). "Inwardly rectifying potassium channels (Kir) in central nervous system glia: a special role for Kir4.1 in glial functions." J Cell Mol Med **10**(1): 33-44.

Byrne, S., et al. (2016). "EPG5-related Vici syndrome: a paradigm of neurodevelopmental disorders with defective autophagy." Brain **139**(Pt 3): 765-781.

Campbell, G. R., et al. (2011). "Mitochondrial DNA deletions and neurodegeneration in multiple sclerosis." Annals of neurology **69**(3): 481-492.

Castanon, I., et al. (2001). "Dimerization partners determine the activity of the Twist bHLH protein during Drosophila mesoderm development." Development **128**(16): 3145-3159.

Cekanaviciute, E., et al. (2014). "Astrocytic transforming growth factor-beta signaling reduces subacute neuroinflammation after stroke in mice." Glia **62**(8): 1227-1240.

Chang, S., et al. (2008). "Identification of small molecules rescuing fragile X syndrome phenotypes in Drosophila." Nat Chem Biol **4**(4): 256-263.

Chaturvedi, R., et al. (2014). "Long-distance mechanism of neurotransmitter recycling mediated by glial network facilitates visual function in Drosophila." Proceedings of the National Academy of Sciences of the United States of America **111**(7): 2812-2817.

Cherry, J. D., et al. (2014). "Neuroinflammation and M2 microglia: the good, the bad, and the inflamed." J Neuroinflammation **11**: 98.

Correale, J. (2014). "The role of microglial activation in disease progression." Mult Scler **20**(10): 1288-1295.

Cullup, T., et al. (2013). "Recessive mutations in EPG5 cause Vici syndrome, a multisystem disorder with defective autophagy." Nat Genet **45**(1): 83-87.

Custer, S. K., et al. (2006). "Bergmann glia expression of polyglutamine-expanded ataxin-7 produces neurodegeneration by impairing glutamate transport." Nature Neuroscience **9**(10): 1302-1311.

Davis, S. M., et al. (2014). "Tailless and Atrophin control Drosophila aggression by regulating neuropeptide signalling in the pars intercerebralis." Nature Communications **5**.

Deneen, B., et al. (2006). "The Transcription Factor NFIA Controls the Onset of Gliogenesis in the Developing Spinal Cord." Neuron **52**(6): 953-968.

Derfuss, T., et al. (2009). "Contactin-2/TAG-1-directed autoimmunity is identified in multiple sclerosis patients and mediates gray matter pathology in animals." Proceedings of the National Academy of Sciences of the United States of America **106**(20): 8302-8307.

Desai, U. A., et al. (2006). "Biologically active molecules that reduce polyglutamine aggregation and toxicity." Hum Mol Genet **15**(13): 2114-2124.

Di Malta, C., et al. (2012). "Astrocyte dysfunction triggers neurodegeneration in a lysosomal storage disorder." Proceedings of the National Academy of Sciences **109**(35): E2334-E2342.

Dietzl, G., et al. (2007). "A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila." Nature **448**(7150): 151-156.

Doherty, J., et al. (2009). "Ensheathing Glia Function as Phagocytes in the Adult Drosophila Brain." Journal of Neuroscience **29**(15): 4768-4781.

Dupont, P., et al. (2012). "Reducing canonical Wingless/Wnt signaling pathway confers protection against mutant Huntingtin toxicity in Drosophila." Neurobiology of Disease **47**(2): 237-247.

Edwards, T. N., et al. (2010). "The functional organisation of glia in the adult brain of Drosophila and other insects." Progress in Neurobiology **90**(4): 471-497.

Fanto, M. (2003). "The tumor-suppressor and cell adhesion molecule Fat controls planar polarity via physical interactions with Atrophin, a transcriptional co-repressor." Development **130**(4): 763-774.

Farmer, W. T., et al. (2016). "Neurons diversify astrocytes in the adult brain through sonic hedgehog signaling." Science **351**(6275): 849-854.

Feltri, M. L., et al. (2015). "How Schwann Cells Sort Axons: New Concepts." Neuroscientist.

Fernandes, A., et al. (2014). "Microglia and inflammation: conspiracy, controversy or control?" Cell Mol Life Sci **71**(20): 3969-3985.

Fernandez-Funez, P., et al. (2000). "Identification of genes that modify ataxin-1-induced neurodegeneration." Nature **408**(6808): 101-106.

Fleming, J. E., et al. (1992). "Role of oxidative stress in Drosophila aging." Mutat Res **275**(3-6): 267-279.

Flici, H., et al. (2014). "Interlocked loops trigger lineage specification and stable fates in the Drosophila nervous system." Nat Commun **5**: 4484.

Freeman, M. R., et al. (2001). "Asymmetric Prospero localization is required to generate mixed neuronal/glial lineages in the Drosophila CNS." Development (Cambridge, England) **128**(20): 4103-4112.

Freeman, M. R., et al. (2006). "Glial cell biology in Drosophila and vertebrates." Trends in Neurosciences **29**(2): 82-90.

Freibaum, B. D., et al. (2015). "GGGGCC repeat expansion in C9orf72 compromises nucleocytoplasmic transport." Nature **525**(7567): 129-133.

Frühbeis, C., et al. (2012). "Emerging Roles of Exosomes in Neuron–Glia Communication." Frontiers in Physiology **3**.

Fulga, T. A., et al. (2015). "A transgenic resource for conditional competitive inhibition of conserved Drosophila microRNAs." Nature Communications **6**: 7279.

Furrer, S. A., et al. (2011). "Spinocerebellar Ataxia Type 7 Cerebellar Disease Requires the Coordinated Action of Mutant Ataxin-7 in Neurons and Glia, and Displays Non-Cell-

Autonomous Bergmann Glia Degeneration." Journal of Neuroscience **31**(45): 16269-16278.

Garden, G. A., et al. (2002). "Polyglutamine-expanded ataxin-7 promotes non-cell-autonomous purkinje cell degeneration and displays proteolytic cleavage in ataxic transgenic mice." J Neurosci **22**(12): 4897-4905.

Glorioso, J. C., et al. (2011). "Targeting Several CAG Expansion Diseases by a Single Antisense Oligonucleotide." PLoS One **6**(9): e24308.

Granderath, S., et al. (2000). "gcm and pointed synergistically control glial transcription of the Drosophila gene loco." Mechanisms of development **91**(1-2): 197-208.

Granderath, S., et al. (1999). "loco encodes an RGS protein required for Drosophila glial differentiation." Development (Cambridge, England) **126**(8): 1781-1791.

Hakim, Y., et al. (2014). "Astrocytes play a key role in Drosophila mushroom body axon pruning." PLoS One **9**(1): e86178.

Halassa, M. M., et al. (2007). "Synaptic islands defined by the territory of a single astrocyte." J Neurosci **27**(24): 6473-6477.

Halter, D. A., et al. (1995). "The homeobox gene repo is required for the differentiation and maintenance of glia function in the embryonic nervous system of Drosophila melanogaster." Development (Cambridge, England) **121**(2): 317-332.

Harris, M. L., et al. (2013). "A dual role for SOX10 in the maintenance of the postnatal melanocyte lineage and the differentiation of melanocyte stem cell progenitors." PLoS Genet **9**(7): e1003644.

Hasegawa, A., et al. (2010). "Long-term disability and prognosis in dentatorubral-pallidoluysian atrophy: A correlation with CAG repeat length." Movement Disorders **25**(11): 1694-1700.

Hashida, H., et al. (2001). "Single cell analysis of CAG repeat in brains of dentatorubral-pallidoluysian atrophy (DRPLA)." Journal of the neurological sciences **190**(1-2): 87-93.

Hatan, M., et al. (2011). "The Drosophila blood brain barrier is maintained by GPCR-dependent dynamic actin structures." The Journal of cell biology **192**(2): 307-319.

Herculano-Houzel, S. (2014). "The glia/neuron ratio: how it varies uniformly across brain structures and species and what that means for brain physiology and evolution." Glia **62**(9): 1377-1391.

Hinz, J., et al. (2011). "Polyglutamine Expansion Alters the Dynamics and Molecular Architecture of Aggregates in Dentatorubropallidoluysian Atrophy." Journal of Biological Chemistry **287**(3): 2068-2078.

Hirrlinger, J., et al. (2014). "Adapting brain metabolism to myelination and long-range signal transduction." Glia **62**(11): 1749-1761.

Hirth, F. (2010). "Drosophila melanogaster in the study of human neurodegeneration." CNS & neurological disorders drug targets **9**(4): 504-523.

Homem, C. C. F., et al. (2012). "Drosophila neuroblasts: a model for stem cell biology." Development **139**(23): 4297-4310.

Hong, S. T., et al. (2006). "Histamine and its receptors modulate temperature-preference behaviors in Drosophila." J Neurosci **26**(27): 7245-7256.

Hong, W., et al. (2009). "Leucine-rich repeat transmembrane proteins instruct discrete dendrite targeting in an olfactory map." Nature Neuroscience **12**(12): 1542-1550.

Hornig, J., et al. (2013). "The transcription factors Sox10 and Myrf define an essential regulatory network module in differentiating oligodendrocytes." PLoS Genet **9**(10): e1003907.

Hosamani, R., et al. (2013). "Acute exposure of Drosophila melanogaster to paraquat causes oxidative stress and mitochondrial dysfunction." Arch Insect Biochem Physiol **83**(1): 25-40.

Hosoya, T., et al. (1995). "glial cells missing: a binary switch between neuronal and glial determination in Drosophila." Cell **82**(6): 1025-1036.

Howe, J. R., et al. (1990). "Sodium currents in Schwann cells from myelinated and non-myelinated nerves of neonatal and adult rabbits." J Physiol **425**: 169-210.

Hu, S., et al. (1998). "Midline Fasciclin: a Drosophila Fasciclin-I-related membrane protein localized to the CNS midline cells and trachea." Journal of neurobiology **35**(1): 77-93.

Hudson, R. S., et al. (2012). "MicroRNA-1 is a candidate tumor suppressor and prognostic marker in human prostate cancer." Nucleic acids research **40**(8): 3689-3703.

Irigoyen, M., et al. (2008). "TGFbeta-induced protein mediates lymphatic endothelial cell adhesion to the extracellular matrix under low oxygen conditions." Cellular and molecular life sciences : CMLS **65**(14): 2244-2255.

Irwin, S., et al. (2005). "RNA association and nucleocytoplasmic shuttling by ataxin-1." J Cell Sci **118**(Pt 1): 233-242.

Izarra, A., et al. (2014). "miRNA-1 and miRNA-133a are involved in early commitment of pluripotent stem cells and demonstrate antagonistic roles in the regulation of cardiac differentiation." J Tissue Eng Regen Med.

Jacques, C., et al. (2009). "A novel role of the glial fate determinant glial cells missing in hematopoiesis." The International Journal of Developmental Biology **53**(7): 1013-1022.

Jha, M. K., et al. (2012). "Glia as a Link between Neuroinflammation and Neuropathic Pain." Immune Network **12**(2): 41.

Johnson, K. M., et al. (2015). "Extracellular matrix composition determines astrocyte responses to mechanical and inflammatory stimuli." Neurosci Lett **600**: 104-109.

Jones, B. W., et al. (1995). "glial cells missing: a genetic switch that controls glial versus neuronal fate." Cell **82**(6): 1013-1023.

Jovičić, A., et al. (2015). "Modifiers of C9orf72 dipeptide repeat toxicity connect nucleocytoplasmic transport defects to FTD/ALS." Nature Neuroscience **18**(9): 1226-1229.

Kang, P., et al. (2012). "Sox9 and NFIA Coordinate a Transcriptional Regulatory Cascade during the Initiation of Gliogenesis." Neuron **74**(1): 79-94.

Kawano, T., et al. (1999). "Cloning and characterization of a Drosophila melanogaster cDNA encoding a glutamate transporter." Biosci Biotechnol Biochem **63**(11): 2042-2044.

Kazemi-Esfarjani, P., et al. (2000). "Genetic suppression of polyglutamine toxicity in Drosophila." Science **287**(5459): 1837-1840.

Kerr, K. S., et al. (2014). "Glial Wingless/Wnt Regulates Glutamate Receptor Clustering and Synaptic Physiology at the Drosophila Neuromuscular Junction." Journal of Neuroscience **34**(8): 2910-2920.

Kim, J., et al. (2010). "Blood-brain barrier defects associated with Rbp9 mutation." Molecules and Cells **29**(1): 93-98.

Kis, V., et al. (2015). "Specialized Cortex Glial Cells Accumulate Lipid Droplets in Drosophila melanogaster." PLoS One **10**(7): e0131250.

Kohsaka, H., et al. (2009). "Target recognition at the tips of postsynaptic filopodia: accumulation and function of Capricious." Development **136**(7): 1127-1135.

Koide, R., et al. (1994). "Unstable expansion of CAG repeat in hereditary dentatorubral-pallidoluysian atrophy (DRPLA)." Nat Genet **6**(1): 9-13.

Koizumi, K., et al. (2007). "RNA interference screen to identify genes required for Drosophila embryonic nervous system development." Proceedings of the National Academy of Sciences of the United States of America **104**(13): 5626-5631.

Kolotuev, I., et al. (2009). "Secretion of Hedgehog-Related Peptides and WNT DuringCaenorhabditis elegansDevelopment." Traffic **10**(7): 803-810.

Komure, O., et al. (1995). "DNA analysis in hereditary dentatorubral-pallidoluysian atrophy: correlation between CAG repeat length and phenotypic variation and the molecular basis of anticipation." Neurology **45**(1): 143-149.

Kozomara, A., et al. (2011). "miRBase: integrating microRNA annotation and deep-sequencing data." Nucleic Acids Res **39**(Database issue): D152-157.

Kozomara, A., et al. (2014). "miRBase: annotating high confidence microRNAs using deep sequencing data." Nucleic acids research **42**(Database issue): D68-73.

Kurusu, M., et al. (2008). "A Screen of Cell-Surface Molecules Identifies Leucine-Rich Repeat Proteins as Key Mediators of Synaptic Target Selection." Neuron **59**(6): 972-985.

La Spada, A. R., et al. (1991). "Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy." Nature **352**(6330): 77-79.

Laneve, P., et al. (2013). "The Gcm/Glide molecular and cellular pathway: New actors and new lineages." Developmental Biology **375**(1): 65-78.

Latouche, M., et al. (2007). "A Conditional Pan-Neuronal Drosophila Model of Spinocerebellar Ataxia 7 with a Reversible Adult Phenotype Suitable for Identifying Modifier Genes." Journal of Neuroscience **27**(10): 2483-2492.

Le Bourg, E. (2001). "Oxidative stress, aging and longevity in Drosophila melanogaster." FEBS letters **498**(2-3): 183-186.

Lee, B. P., et al. (2005). "Transcriptional regulation of the Drosophila glial gene repo." Mechanisms of development **122**(6): 849-862.

Lee, R. C., et al. (2001). "An extensive class of small RNAs in Caenorhabditis elegans." Science **294**(5543): 862-864.

Lee, Y., et al. (2012). "Oligodendroglia metabolically support axons and contribute to neurodegeneration." Nature **487**(7408): 443-448.

Lee, Y. M., et al. (2015). "Drosophila as a model to study the role of glia in neurodegeneration." J Neurogenet **29**(2-3): 69-79.

Levine, B. D., et al. (2016). "Drosophila Lung Cancer Models Identify Trametinib plus Statin as Candidate Therapeutic." Cell Rep.

Li, S. H., et al. (1993). "Novel triplet repeat containing genes in human brain: cloning, expression, and length polymorphisms." Genomics **16**(3): 572-579.

Liévens, J. C., et al. (2001). "Impaired Glutamate Uptake in the R6 Huntington's Disease Transgenic Mice." Neurobiology of Disease **8**(5): 807-821.

Lim, L. P., et al. (2005). "Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs." Nature **433**(7027): 769-773.

Limmer, S., et al. (2014). "The Drosophila blood-brain barrier: development and function of a glial endothelium." Front Neurosci **8**: 365.

Lin, Y. R., et al. (2011). "Regulation of longevity by regulator of G-protein signaling protein, Loco." Aging Cell **10**(3): 438-447.

Lisak, R. P., et al. (2009). "Differential effects of Th1, monocyte/macrophage and Th2 cytokine mixtures on early gene expression for molecules associated with metabolism, signaling and regulation in central nervous system mixed glial cell cultures." Journal of neuroinflammation **6**: 4.

Liu, L., et al. (2015). "Glial Lipid Droplets and ROS Induced by Mitochondrial Defects Promote Neurodegeneration." Cell **160**(1-2): 177-190.

Loftus, S. K., et al. (2002). "Rescue of neurodegeneration in Niemann-Pick C mice by a prion-promoter-driven Npc1 cDNA transgene." Hum Mol Genet **11**(24): 3107-3114.

Luthi-Carter, R., et al. (2002). "Polyglutamine and transcription: gene expression changes shared by DRPLA and Huntington's disease mouse models reveal context-independent effects." Hum Mol Genet **11**(17): 1927-1937.

Magistretti, P. J. (2006). "Neuron-glia metabolic coupling and plasticity." J Exp Biol **209**(Pt 12): 2304-2311.

Malatesta, P., et al. (2000). "Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage." Development **127**(24): 5253-5263.

Margolis, R. L., et al. (1993). Spinocerebellar Ataxia Type 12. GeneReviews(R). R. A. Pagon et al. Seattle (WA).

Marsh, J. L., et al. (2000). "Expanded polyglutamine peptides alone are intrinsically cytotoxic and cause neurodegeneration in Drosophila." Hum Mol Genet **9**(1): 13-25.

Matsuno, M., et al. (2015). "Long-Term Memory Formation in Drosophila Requires Training-Dependent Glial Transcription." Journal of Neuroscience **35**(14): 5557-5565.

McCabe, B. D., et al. (2015). "A Distinct Perisynaptic Glial Cell Type Forms Tripartite Neuromuscular Synapses in the Drosophila Adult." PLoS One **10**(6): e0129957.

McGurk, L., et al. (2015). "Drosophila as an In Vivo Model for Human Neurodegenerative Disease." Genetics **201**(2): 377-402.

Mitchell, K. J., et al. (1996). "Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons." Neuron **17**(2): 203-215.

Morante, J., et al. (2004). "Building a projection map for photoreceptor neurons in the Drosophila optic lobes." Seminars in cell & developmental biology **15**(1): 137-143.

Muñoz-Soriano, V., et al. (2011). "Drosophila Models of Parkinson's Disease: Discovering Relevant Pathways and Novel Therapeutic Strategies." Parkinson's Disease **2011**: 1-14.

Muqit, M. M. K., et al. (2002). "Modelling neurodegenerative diseases in Drosophila: a fruitful approach?" Nature reviews Neuroscience **3**(3): 237-243.

Muthukumar, A. K., et al. (2014). "Activity-dependent regulation of astrocyte GAT levels during synaptogenesis." Nature Neuroscience **17**(10): 1340-1350.

Nagafuchi, S., et al. (1994). "Dentatorubral and pallidoluysian atrophy expansion of an unstable CAG trinucleotide on chromosome 12p." Nature genetics **6**(1): 14-18.

Nagelhus, E. A., et al. (2004). "Aquaporin-4 in the central nervous system: cellular and subcellular distribution and coexpression with KIR4.1." Neuroscience **129**(4): 905-913.

Namihira, M., et al. (2009). "Committed neuronal precursors confer astrocytic potential on residual neural precursor cells." Dev Cell **16**(2): 245-255.

Napoletano, F., et al. (2011). "Polyglutamine Atrophin provokes neurodegeneration inDrosophilaby repressingfat." The EMBO Journal **30**(5): 945-958.

Nisoli, I., et al. (2010). "Neurodegeneration by polyglutamine Atrophin is not rescued by induction of autophagy." Cell Death and Differentiation **17**(10): 1577-1587.

Norden, D. M., et al. (2014). "TGFβ produced by IL-10 redirected astrocytes attenuates microglial activation." Glia **62**(6): 881-895.

Nucifora Jr, F. C. (2001). "Interference by Huntingtin and Atrophin-1 with CBP-Mediated Transcription Leading to Cellular Toxicity." Science **291**(5512): 2423-2428.

Orr, H. T. (2012). "SCA1-phosphorylation, a regulator of Ataxin-1 function and pathogenesis." Progress in Neurobiology **99**(3): 179-185.

Orr, H. T., et al. (2007). "Trinucleotide repeat disorders." Annual review of neuroscience **30**: 575-621.

Pannasch, U., et al. (2014). "Connexin 30 sets synaptic strength by controlling astroglial synapse invasion." Nature Neuroscience **17**(4): 549-558.

Pantazis, A., et al. (2008). "Distinct roles for two histamine receptors (hclA and hclB) at the Drosophila photoreceptor synapse." The Journal of neuroscience : the official journal of the Society for Neuroscience **28**(29): 7250-7259.

Paradis, S., et al. (2007). "An RNAi-based approach identifies molecules required for glutamatergic and GABAergic synapse development." Neuron **53**(2): 217-232.

Park, J., et al. (2009). "Mitochondrial dysfunction and Parkinson's disease genes: insights from Drosophila." Disease Models & Mechanisms **2**(7-8): 336-340.

Parker, R. J., et al. (2006). "Roles of glia in the Drosophila nervous system." Seminars in cell & developmental biology **17**(1): 66-77.

Parkhurst, C. N., et al. (2013). "Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor." Cell **155**(7): 1596-1609.

Peco, E., et al. (2016). "Drosophila astrocytes cover specific territories of CNS neuropil and are instructed to differentiate by Prospero, a key effector of Notch." Development.

Pekny, M., et al. (2005). "Astrocyte activation and reactive gliosis." Glia **50**(4): 427-434.

Rasband, M. N. (2015). "Glial contributions to neural function and disease." Mol Cell Proteomics.

Risher, W. C., et al. (2012). "Thrombospondins as key regulators of synaptogenesis in the central nervous system." Matrix Biology **31**(3): 170-177.

Rival, T., et al. (2004). "Decreasing Glutamate Buffering Capacity Triggers Oxidative Stress and Neuropil Degeneration in the Drosophila Brain." Current Biology **14**(7): 599-605.

Rowitch, D. H., et al. (2010). "Developmental genetics of vertebrate glial-cell specification." Nature **468**(7321): 214-222.

Rubinsztein, D. C., et al. (1993). "Analysis of the huntingtin gene reveals a trinucleotide-length polymorphism in the region of the gene that contains two CCG-rich stretches and a correlation between decreased age of onset of Huntington's disease and CAG repeat number." Human Molecular Genetics **2**(10): 1713-1715.

Sakai, K. (2006). "Neuronal atrophy and synaptic alteration in a mouse model of dentatorubral-pallidoluysian atrophy." Brain **129**(9): 2353-2362.

Sargin, D., et al. (2009). "Uncoupling of neurodegeneration and gliosis in a murine model of juvenile cortical lesion." Glia **57**(7): 693-702.

Sato, T., et al. (2008). "Severe neurological phenotypes of Q129 DRPLA transgenic mice serendipitously created by en masse expansion of CAG repeats in Q76 DRPLA mice." Human Molecular Genetics **18**(4): 723-736.

Sato, T., et al. (1999). "Transgenic mice harboring a full-length human mutant DRPLA gene exhibit age-dependent intergenerational and somatic instabilities of CAG repeats comparable with those in DRPLA patients." Human Molecular Genetics **8**(1): 99-106.

Schafer, D. P., et al. (2012). "Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner." Neuron **74**(4): 691-705.

Schilling, G., et al. (2001). "Distinct behavioral and neuropathological abnormalities in transgenic mouse models of HD and DRPLA." Neurobiol Dis **8**(3): 405-418.

Schilling, G., et al. (1999). "Nuclear accumulation of truncated atrophin-1 fragments in a transgenic mouse model of DRPLA." Neuron **24**(1): 275-286.

Schirmeier, S., et al. (2015). "The Drosophila blood-brain barrier as interface between neurons and hemolymph." Mechanisms of development.

Schirmeier, S., et al. (2015). "Axon ensheathment and metabolic supply by glial cells in Drosophila." Brain Research.

Schitine, C., et al. (2015). "Astrocyte heterogeneity in the brain: from development to disease." Frontiers in Cellular Neuroscience **9**.

Schuster, C. M., et al. (1996). "Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth." Neuron **17**(4): 641-654.

Schwab, C., et al. (2010). "Inflammation in transgenic mouse models of neurodegenerative disorders." Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease **1802**(10): 889-902.

Schwabe, T., et al. (2005). "GPCR Signaling Is Required for Blood-Brain Barrier Formation in Drosophila." Cell **123**(1): 133-144.

Seal, R. P., et al. (1998). "Identification and characterization of a cDNA encoding a neuronal glutamate transporter from Drosophila melanogaster." Receptors Channels **6**(1): 51-64.

Selby, L., et al. (2007). "Major defects in neocortical GABAergic inhibitory circuits in mice lacking the fragile X mental retardation protein." Neurosci Lett **412**(3): 227-232.

Sempere, L. F., et al. (2004). "Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation." Genome Biol **5**(3): R13.

Sharma, K., et al. (2012). "Scar-mediated inhibition and CSPG receptors in the CNS." Exp Neurol **237**(2): 370-378.

Shin, J.-Y., et al. (2005). "Expression of mutant huntingtin in glial cells contributes to neuronal excitotoxicity." The Journal of cell biology **171**(6): 1001-1012.

Shiozawa, M., et al. (2003). "Glyceraldehyde 3-phosphate dehydrogenase and endothelin-1 immunoreactivity is associated with cerebral white matter damage in dentatorubral-pallidoluysian atrophy." Neuropathology : official journal of the Japanese Society of Neuropathology **23**(1): 36-43.

Shishido, E. (1998). "Drosophila Synapse Formation: Regulation by Transmembrane Protein with Leu-Rich Repeats, CAPRICIOUS." Science **280**(5372): 2118-2121.

Skonier, J., et al. (1994). "beta ig-h3: a transforming growth factor-beta-responsive gene encoding a secreted protein that inhibits cell attachment in vitro and suppresses the growth of CHO cells in nude mice." DNA and cell biology **13**(6): 571-584.

Slack, C., et al. (2011). "dFOXO-independent effects of reduced insulin-like signaling in Drosophila." Aging Cell **10**(5): 735-748.

Sofroniew, M. V. (2015). "Astrocyte barriers to neurotoxic inflammation." Nat Rev Neurosci **16**(5): 249-263.

Sokol, N. S. (2005). "Mesodermally expressed Drosophila microRNA-1 is regulated by Twist and is required in muscles during larval growth." Genes & Development **19**(19): 2343-2354.

Sousa-Nunes, R., et al. (2011). "Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in Drosophila." Nature **471**(7339): 508-512.

Sousa, I., et al. (2010). "Polymorphisms in leucine-rich repeat genes are associated with autism spectrum disorder susceptibility in populations of European ancestry." Molecular Autism **1**(1): 7.

Soustelle, L., et al. (2002). "Terminal Glial Differentiation Involves Regulated Expression of the Excitatory Amino Acid Transporters in the Drosophila Embryonic CNS." Developmental Biology **248**(2): 294-306.

Soustelle, L., et al. (2007). "Novel gcm-dependent lineages in the postembryonic nervous system of Drosophila melanogaster." Dev Dyn **236**(8): 2101-2108.

Spéder, P., et al. (2014). "Gap Junction Proteins in the Blood-Brain Barrier Control Nutrient-Dependent Reactivation of Drosophila Neural Stem Cells." Developmental Cell **30**(3): 309-321.

Spindler, S. R., et al. (2009). "Drosophila cortex and neuropile glia influence secondary axon tract growth, pathfinding, and fasciculation in the developing larval brain." Dev Biol **334**(2): 355-368.

Stork, T., et al. (2014). "Neuron-Glia Interactions through the Heartless FGF Receptor Signaling Pathway Mediate Morphogenesis of Drosophila Astrocytes." Neuron **83**(2): 388-403.

Su, F.-C., et al. (2012). "LINGO-2 polymorphism and the risk of Parkinson's disease in Taiwan." Parkinsonism & Related Disorders **18**(5): 609-611.

Sunami, Y., et al. (2010). "Radiologic and Neuropathologic Findings in Patients in a Family with Dentatorubral-Pallidoluysian Atrophy." American Journal of Neuroradiology.

Suzuki, K., et al. (2012). "DRPLA transgenic mouse substrains carrying single copy of full-length mutant human DRPLA gene with variable sizes of expanded CAG repeats exhibit CAG repeat length- and age-dependent changes in behavioral abnormalities and gene expression profiles." Neurobiology of Disease **46**(2): 336-350.

Tang, F., et al. (2014). "Lactate-mediated glia-neuronal signalling in the mammalian brain." Nat Commun **5**: 3284.

Tasdemir-Yilmaz, O. E., et al. (2013). "Astrocytes engage unique molecular programs to engulf pruned neuronal debris from distinct subsets of neurons." Genes & Development **28**(1): 20-33.

Thomas, E. A., et al. (2010). "In vivo cell-autonomous transcriptional abnormalities revealed in mice expressing mutant huntingtin in striatal but not cortical neurons." Human Molecular Genetics **20**(6): 1049-1060.

Thomas, G. B., et al. (2006). "The glycosyltransferase Fringe promotes Delta-Notch signaling between neurons and glia, and is required for subtype-specific glial gene expression." Development **134**(3): 591-600.

Tran, H., et al. (2015). "Differential Toxicity of Nuclear RNA Foci versus Dipeptide Repeat Proteins in a Drosophila Model of C9ORF72 FTD/ALS." Neuron **87**(6): 1207-1214.

Trapp, B. D., et al. (1998). "Axonal transection in the lesions of multiple sclerosis." N Engl J Med **338**(5): 278-285.

Tsuyama, J., et al. (2015). "MicroRNA-153 Regulates the Acquisition of Gliogenic Competence by Neural Stem Cells." Stem Cell Reports **5**(3): 365-377.

Vaccari, T., et al. (2010). "The vacuolar ATPase is required for physiological as well as pathological activation of the Notch receptor." Development **137**(11): 1825-1832.

Valakh, V., et al. (2012). "A large-scale RNAi screen identifies functional classes of genes shaping synaptic development and maintenance." Dev Biol **366**(2): 163-171.

Vincent, S., et al. (1996). "Glide directs glial fate commitment and cell fate switch between neurones and glia." Development (Cambridge, England) **122**(1): 131-139.

Volkenhoff, A., et al. (2015). "Glial Glycolysis Is Essential for Neuronal Survival in Drosophila." Cell Metabolism **22**(3): 437-447.

Warner, T. T., et al. (1994). "DRPLA in Europe." Nat Genet **6**(3): 225.

Wong, P. C., et al. (2002). "Genetically engineered mouse models of neurodegenerative diseases." Nat Neurosci **5**(7): 633-639.

Xiong, W. C., et al. (1995). "Defective glia induce neuronal apoptosis in the repo visual system of Drosophila." Neuron **14**(3): 581-590.

Xiong, W. C., et al. (1994). "repo encodes a glial-specific homeo domain protein required in the Drosophila nervous system." Genes & Development **8**(8): 981-994.

Yagi, R., et al. (2010). "Refined LexA transactivators and their use in combination with the Drosophila Gal4 system." Proceedings of the National Academy of Sciences of the United States of America **107**(37): 16166-16171.

Yamada, M., et al. (2002). "Oligodendrocytic polyglutamine pathology in dentatorubral-pallidoluysian atrophy." Annals of neurology **52**(5): 670-674.

Yazawa, I. (2000). "Aberrant phosphorylation of dentatorubral-pallidoluysian atrophy (DRPLA) protein complex in brain tissue." Biochem J **351 Pt 3**: 587-593.

Yazawa, I., et al. (1995). "Abnormal gene product identified in hereditary dentatorubral-pallidoluysian atrophy (DRPLA) brain." Nat Genet **10**(1): 99-103.

Ying, M., et al. (2005). "Sodium Butyrate Ameliorates Histone Hypoacetylation and Neurodegenerative Phenotypes in a Mouse Model for DRPLA." Journal of Biological Chemistry **281**(18): 12580-12586.

Yuan, Y. M., et al. (2013). "The glial scar in spinal cord injury and repair." Neurosci Bull **29**(4): 421-435.

Yuasa, Y. (2003). "Drosophila homeodomain protein REPO controls glial differentiation by cooperating with ETS and BTB transcription factors." Development **130**(11): 2419-2428.

Zhang, K., et al. (2015). "The C9orf72 repeat expansion disrupts nucleocytoplasmic transport." Nature **525**(7567): 56-61.

Zhang, S., et al. (2002). "Drosophila atrophin homolog functions as a transcriptional corepressor in multiple developmental processes." Cell **108**(1): 45-56.

Zhang, X., et al. (2005). "A potent small molecule inhibits polyglutamine aggregation in Huntington's disease neurons and suppresses neurodegeneration in vivo." Proceedings of the National Academy of Sciences of the United States of America **102**(3): 892-897.

Zhou, Z. D., et al. (2012). "LINGO-1 and Neurodegeneration: Pathophysiologic Clues for Essential Tremor." Tremor Other Hyperkinet Mov (N Y) **2**.

Zoghbi, H. Y., et al. (2000). "Glutamine repeats and neurodegeneration." Annual review of neuroscience **23**: 217-247.

Zwarts, L., et al. (2014). "Glia in Drosophila behavior." Journal of Comparative Physiology A **201**(9): 879-893.